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STUDY DOCUMENT E

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Study Title

(*Agrobacterium radiobacter* strain K84) -- Tier 1 Tox, Nontarget organisms

Data Requirement

Guideline 154A-19 and 154A-22

Author

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Study Completed On

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Performing Laboratory

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Laboratory Project ID

L.W.M.-5

Facts of Publication

References from the literature

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1 874

## STATEMENT OF DATA CONFIDENTIALITY

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA S10(d) (1) (A), (B), or (C).

Company Agent: Larry W. Moore

Date: 9/26/90

       Title: President

Signature: Larry W. Moore

## CERTIFICATION OF GOOD LABORATORY PRACTICE

The submitter of this study neither sponsored nor conducted all the research reported in this study. Part of the sponsor's work was performed before 40 CFR-160 was promulgated, as was the other work cited herein. Although only part of the information I am providing was collected according to present good laboratory practice requirements, I feel the work is valid and acceptable scientifically. In each literature citation, the work cited was published in reputable, referred scientific journals.

## BODY OF STUDY

### Tier 1 Tox

Guideline Reference Numbers: 154A-19. I will rely on the data from the earlier data submission which is on record with EPA.

Again, the maximum number of CFU of Agrobacterium radiobacter K84 administered in these tests, without causing toxicity, far exceeds the concentration with which fish would be subjected were K84 to enter an aquatic system, especially from run-off water from a field with treated plants. Furthermore, the populations of wild Agrobacterium spp. naturally present in the soil or on roots would be as great or greater than K84 [Appendix items 1 (Table 1); 2 (P. 113); 3 (Table 3)]. Taxonomically, K84 is ..... indistinguishable from other Agrobacterium biovar 2 strains and also from the fast... growing Rhizobium spp. (cf. Appendix item # 4; even though Skinner suggests that... Nile Blue differentiates the rhizobia from Agrobacterium, cf. Appendix item # 5, p. 16), and K84 would therefore contribute but to but a fraction of the agrobacteria entering the aquatic system. Although we have isolated biovar 2 agrobacteria from streams onto a selective medium for biovar 2 strains, an antibiotic-resistant mutant strain of K84 did not survive well in water from the Willamette and Marys Rivers near Corvallis (cf. Appendix item # 6; Stockwell, V., and Moore, L. W., manuscript in preparation). These considerations plus: (i) the relatively small amount of K84 used in agriculture, (ii) the very targeted application of K84 directly to the plant part to be protected rather than to soil, water, or an aerial spray of foliage, and (iii) the use of K84 on minor non-food type plants grown in a nursery or landscape are major reasons for my position on these questions.

Guideline Reference Number: 154A-22 K84 survived on roots of a dozen nontarget plants (broadleaf weeds, grasses, etc.--cf. Appendix item # 6). Survival on roots of nontarget plants was generally good overall, but better on some species. Survival in the rhizosphere of nontarget plants is not surprising since the agrobacteria are rhizosphere inhabitants. Again, K84 is only one component of the total Agrobacterium population in these niches. Using annual rye grass as trap plants, we could monitor movement of an antibiotic marked strain of K84 from a point source (inoculated cherry seedling) to grass plants 40 cm. from the inoculum source, but movement in natural, mineral soil was never greater than 5 cm and survival was relatively short (cf. Appendix item # 6; Stockwell, V., and Moore, L. W., manuscript in preparation).

The agrobacteria are eaten by nematodes (cf. Appendix item # 7) and are likely carried on the body of arthropods and beetles which reside in soil and on plant roots. The importance of these potential vectors in helping K84 become established in different niches within the ecosystem at sites distant from the original source of K84 is unknown, but I predict it would be small. Once K84 is disseminated from a point source to a new area, it faces strong competition from other microorganisms relative to establishment.

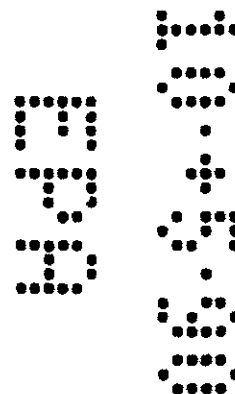
#### STUDY DOCUMENT E APPENDIX

##### ITEM NO.

##### REFERENCE

- 1 Bouzar, H. and L. W. Moore. 1987. Isolation of different Agrobacterium biovars from a natural oak savanna and tallgrass prairie. Appl. Environ. Microbiol. 53: 717-721.
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- 4 Anderson, A. R. 1978. Taxonomy and Host Specificity of the Genus Agrobacterium. Ph.D. Thesis, Department of Botany and Plant Pathology, Oregon State University, 61 pp.
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- 6 Moore, L., Stockwell, V., Loper, J., and Kawalek, M. 1990. Environmental fate of *Agrobacterium radiobacter* K84 released in agricultural fields. in: Review of Progress in the Biotechnology-Microbial Pest Control Agent Risk Assessment Program. USEPA/600/9-90/029, pp.125-130.
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Study Doc. E-1

## Isolation of Different *Agrobacterium* Biovars from a Natural Oak Savanna and Tallgrass Prairie†

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Populations of *agrobacteria* in excess of  $10^5$  CFU/g were recovered from 12 soil and root samples obtained from the Allison Savanna, Minn., a natural oak savanna and tallgrass prairie which has never been disturbed agriculturally. Of 126 strains picked randomly from selective media, 54 were identified as *Agrobacterium* spp. Biovar 2 strains predominated (35 of 54), but these strains were distributed into three phenotypically distinct subgroups. Of the remaining *Agrobacterium* strains, four were biovar 1-2, one was biovar 1, and none were biovar 3. The last 14 *Agrobacterium* strains formed a homogeneous group which differed biochemically from the hitherto reported biovars. Opine utilization (coded for by genes on the tumor-inducing plasmid in pathogenic *Agrobacterium* spp.) by these *agrobacteria* was limited to two biovar 2 strains. In contrast, 10 nonfluorescent gram-negative strains utilized either nopaline or octopine as the sole carbon and nitrogen source. There may be a need to reexamine the source and role of opines in the terrestrial environment because (i) all of these opine utilizers were isolated from an environment free of crown gall, the only known terrestrial source of opines, and (ii) 83% of the opine utilizers were not *Agrobacterium* spp.

The ecology of *Agrobacterium* spp. in nonagricultural environments has received little attention, partly because the research by plant pathologists has focused primarily on crown gall, a serious neoplastic disease in plant nurseries caused by pathogenic *agrobacteria* (28). Such research has shown that *Agrobacterium* spp. are able to successfully invade cultivated soils and cause epidemics (8, 33). Yet in these cultivated soils, the *Agrobacterium* population is predominantly nonpathogenic (15, 33). Although nonpathogenic strains could potentially become pathogens upon acquisition of the conjugative Ti plasmid (pTi) (39), which is responsible for tumor induction (40), most plant pathologists do not consider the nonpathogenic population to be a threat to the development of crown gall epidemics. This perception may be the reason why the biological activity and survival of this organism in nonagricultural soils has not been investigated.

The present study was designed to determine whether *Agrobacterium* strains inhabit soils of a natural environment and, if so, whether their phenotypic characteristics would associate them with the presently recognized biovars (19). For this purpose, soil and root samples were obtained from the Allison Savanna, Minn., a natural oak savanna and tallgrass prairie which has never been disturbed agriculturally. This preserve has been maintained as it was in presettlement days, when such plant communities covered much of the central North American continent.

This paper reports the range of *Agrobacterium* occurrence in the Allison Savanna, the predominance of biovar 2 strains in root and soil samples obtained from this savanna, a hitherto undescribed physiological group of *agrobacteria*, and the predominance among opine utilizers of bacteria that are not *Agrobacterium* spp.

### MATERIALS AND METHODS

**Organisms and cultivation.** Bacteria were isolated from both root and soil surrounding plants in the families

Fagaceae (*Quercus* sp.), Rosaceae (*Rubus flagellaris*), Fabaceae (*Lathyrus venosus*), Asteraceae, and Poaceae and in an unidentified moss plant (class Muscopsida). All plants sampled were located several hundred meters apart in the Allison Savanna, a preserve of The Nature Conservancy, located in the Anoka Sand Plain of Anoka County, Minn. Individual plants with about 250 g of sandy-loam soil surrounding the root system were placed in plastic bags for transport to the laboratory. Roots were removed and washed free of adhering soil, and 1 g of root tissue was ground with a mortar and pestle for 2 min in 10 ml of sterile distilled water. For the soil samples, 1 g of soil was suspended in 10 ml of sterile distilled water and vortexed for 1 min. The soil suspension was passed through a screen with sieve openings of 0.125 mm to remove plant residues. The six root and six soil suspensions were allowed to settle at room temperature for 5 min before 10-fold serial dilutions of each suspension were made; 0.1 ml of selected dilutions was spread onto IA, 2E, and 3DG semiselective media for isolation of *Agrobacterium* spp. (6). The inoculated plates were incubated for 1 week at 25°C before population counts were made. A total of 30 colonies (30 per each medium), representing different colony types on the semiselective media, were selected at random from each sample and purified by two successive streakings on potato dextrose agar (PDA) (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (wt/vol) calcium carbonate. Bacterial cultures were stored at 4°C on PDA-calcium carbonate slants and in sterile distilled water.

**Characterization of strains.** Strains were examined for their Gram reaction (32) and production of fluorescent pigment on King medium B (21). To determine the biovar affiliation (14, 17, 35) of each putative *Agrobacterium* strain, we performed the following tests: oxidation of lactose to 3-ketolactose (3); oxidase reaction (22); growth and pigmentation in ferric ammonium citrate (12); utilization of citrate (34), L-tyrosine (20), mucic acid, and L-(+)-tartaric acid (37); alkali production from malonate (23); and acid production from erythritol and melezitose (17).

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† Technical paper 7987 of the Oregon State University Agriculture Experiment Station.

TABLE 1. Population levels of *Agrobacterium* strains isolated from samples of plant roots and surrounding soil obtained from the Allison Savanna

Colony type <sup>a</sup>	Population levels (10 <sup>5</sup> CFU/g) of <i>Agrobacterium</i> strains from:											
	Moss		Poaceae		Asteraceae		Fabaceae		Fagaceae		Rosaceae	
	Soil	Roots	Soil	Roots	Soil	Roots	Soil	Roots	Soil	Roots	Soil	Roots
1	— <sup>b</sup>	—	—	—	—	1	—	—	—	—	—	—
2	0.2	0.5	30	80	1	6	100	200	10	2	—	1
1-2	—	—	2	—	—	—	—	—	—	—	40	20
4	1	2	—	90	—	—	—	—	5	—	—	—

<sup>a</sup> Four major distinct colony types were observed on the selective media used for isolation of *Agrobacterium* strains. All four types were pulvinate, smooth, glossy, and circular with an entire margin. The type 1 colony was 4 mm in diameter and gray with an orange center on medium 1A. Type 2 colonies were 3 mm in diameter, and consisted of three subgroups: type 2', translucent gray on medium 1A; type 2'', translucent green on medium 2E; type 2''', pink on medium 3DG (all of the type 2 colonies were characterized as physiological group 2). The type 1-2 colony was 3 mm in diameter and pale green on medium 2E. The type 4 colony was about 1 mm in diameter and gray on medium 1A. These colony types correspond to physiological groups shown in Table 2.

<sup>b</sup> —, Not detected at a 10<sup>-2</sup> dilution.

Utilization of opine as the sole carbon and nitrogen source was determined initially from growth on basal medium solidified with 0.4% (wt/vol) Gelrite (Kelco Division of Merck & Co., Inc., San Diego, Calif.) containing 5 mM octopine (Sigma Chemical Co., St. Louis, Mo.) or nopaline (Sigma) and confirmed subsequently by growth in liquid basal medium containing the same opine concentration. Gelrite was used instead of agar as the gelling agent because the use of agar can result in false-positive data (M. L. Canfield and L. W. Moore, unpublished results). The basal medium consisted of the following: K<sub>2</sub>HPO<sub>4</sub>, 41 mM; KH<sub>2</sub>PO<sub>4</sub>, 21 mM; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.8 mM; CaCl<sub>2</sub> · 2H<sub>2</sub>O, 75 μM; FeSO<sub>4</sub> · 7H<sub>2</sub>O, 18 μM; and MnCl<sub>2</sub> · 4H<sub>2</sub>O, 10 μM; the pH was adjusted to 7.2 (Canfield and Moore, unpublished results).

Because some of the putative *Agrobacterium* strains recovered from selective media might be *Rhizobium* strains we inoculated these strains into three species of legumes to test for nodulation. Seeds of alfalfa (*Medicago sativa*), bean (*Phaseolus vulgaris* 'Yellow Wax'), and pea (*Pisum sativum* 'Sugar Snap') were surface sterilized in 70% ethanol for 1 min and in 1% sodium hypochlorite for 10 min, germinated for 2 days at 25°C, and transferred aseptically to sterile test tubes (30 by 3 cm) containing 20 ml of a mineral salts agar medium (9). The seedlings were inoculated with 0.2 ml of bacterial suspension (10<sup>8</sup> CFU/ml) in a growth chamber for 6 weeks before the plants were scored for nodulation. *Rhizobium leguminosarum* biovar *phaseoli* 127K12b, *R. leguminosarum* biovar *trifolii* 16337a, and *R. meliloti* YA-15 were used as positive controls, and sterile distilled water was used as a negative control.

To confirm the identity of the putative *Agrobacterium* strains, purified ribosomes extracted from seven representative strains were reacted in gel immunodiffusion tests with five antisera made against 50S ribosomal subunits of *Agrobacterium* strains B6, C58, W63/79, U11, and CG64 (5).

Pathogenicity tests were performed on stems of 4-week-old seedlings of tomato (*Lycopersicon esculentum* 'Bonny Best') (1), and tumor formation was recorded 8 weeks after inoculation.

Sensitivity to agrocin 84, an antibiotic produced by the biocontrol agent *Agrobacterium* strain K84, was tested by the method of Stonier as modified by Cooksey and Moore (7). Because the genes for agrocin sensitivity are located on the nopaline pTi, this test is helpful as a putative test for pathogenicity (16).

*Agrobacterium* strains B6 (biovar 1, octopine utilizer) (R. Baker, Colorado State University), C58 (biovar 1, nopaline utilizer) (R. Dickey, Cornell University), K84 (biovar 2) (A.

Kerr, Waite Institute, South Australia) and CG64 (biovar 3) (T. Burr, New York State Agriculture Experiment Station at Geneva) were included as reference strains.

## RESULTS

**Isolation of agrobacteria from both soil and root samples.** *Agrobacterium* strains were recovered from all 12 soil and root samples (Table 1). The population of *Agrobacterium* strains in soil ranged from 10<sup>5</sup> to 10<sup>7</sup> CFU/g and was unexpectedly similar to the population on the roots.

**Identification of strains.** From the three different selective media, 126 bacterial colonies representing all different colony types were selected for further characterization. Fifty-four *Agrobacterium* strains were identified, but none was tumorigenic on tomato seedling nor sensitive to agrocin 84.

Thirty-five strains were biovar 2 agrobacteria, one was biovar 1 and four shared characteristics of biovar 1-2 of Spiers (35). The remaining 14 strains formed a fourth physiological group (Table 2). None of the 12 samples yielded biovar 3 strains.

Although the biovar 2 population was predominant in this study, only 8 of 35 biovar 2 strains were isolated on the biovar 2 semiselective medium 2E. Seventeen of these strains which were unable to grow on medium 2E did not grow on erythritol; they formed a biovar 2 subgroup represented by strain R3/84 (Table 2). Another biovar 2 subgroup, represented by strain S2/84, included 15 L-tyrosine-negative strains. A third biovar 2 subgroup consisted of three strains that shared biovar 1 characteristics; they grew on medium 1A and on ferric ammonium citrate. Biovar 2-2 strains differed from the reference biovar 3 strain by their ability to grow on medium 1A and to utilize erythritol; none of these strains did not grow on biovar 3 medium 3DG.

**Ribosomal serology and nodulation assay.** Ribosomes from representative strains of each phenotypic group listed in Table 2 reacted positively in immunodiffusion tests with *Agrobacterium* ribosomal antisera. These strains produced a precipitin band which formed a reaction of complete fusion (identity) with the precipitin band of the homolog (i.e., the strain used to develop the antiserum) (Fig. 1). Results of this serological test and the lack of nodulation of the three legume species confirmed that these strains were agrobacteria.

**Utilization of opines.** Of 126 strains tested, 30 (6 of which were agrobacteria) grew on solid media containing either nopaline or octopine as the sole carbon and nitrogen source (Table 3). However, only 12 (two of which were *Agrobacterium* biovar 2) of the 30 strains grew when they

TABLE 2. Characteristics and distribution of the 54 *Agrobacterium* strains isolated from soil or plant roots from the Allison Savanna

Diagnostic test	Reaction to test of strains in physiological groups <sup>a</sup>									
	1	2	3	1-2	4					
	R6 <sup>b</sup>	R6/84 <sup>c</sup> (1) <sup>d</sup>	K84 <sup>e</sup>	S2/84 (15)	R1/84 (3)	R3/84 (17)	CG64 <sup>f</sup>	R5/84 (3)	S5/84 (1)	S4/84 (14)
3-Ketolactose	+	+	-	-	-	-	-	-	-	-
Oxidase	+	+	-	-	-	-	+	+	+	-
Ferric ammonium citrate	+	+	-	-	+	-	-	-	-	-
Citrate	-	+	+	+	+	+	+	+	+	+
L-Tyrosine	-	-	+	-	+	+	-	-	+	-
Mucic acid	-	-	+	+	+	+	-	-	-	-
L-(+)-Tartaric acid	-	-	+	+	+	+	+	+	+	-
Malonate	-	-	+	+	+	+	-	-	-	-
Erythritol	-	-	+	+	+	+	-	+	-	+
Melzitose	+	+	-	-	-	-	-	-	-	-
Growth on medium 1A	+	+	-	-	+	+	-	+	+	+
Growth on medium 2E	-	-	+	+	-	-	-	+	+	+
Growth on medium 3DG	-	-	-	-	+	+	+	-	+	+

<sup>a</sup> Physiological groups 1, 2, 3, and 1-2 are equivalent to biovars 1, 2, 3 and 1-2 (6, 35).

<sup>b</sup> Reference strain for that physiological group.

<sup>c</sup> Type strain of a group of *Agrobacterium* strains isolated from the Allison Savanna sharing the same phenotype.

<sup>d</sup> Number of strains in that phenotypic group (see footnote b).

<sup>e</sup> Nine of the fourteen strains gave the indicated result.

<sup>f</sup> Thirteen of the fourteen strains gave the indicated result.

were added to a minimal liquid medium containing either opine as the sole carbon and nitrogen source. *Agrobacterium* strain R3/84 utilized octopine, and *Agrobacterium* strain S2/84 utilized separately both nopaline and octopine. The other opine utilizers were gram-negative bacteria which failed to produce a fluorescent pigment on King medium B; seven of these strains were oxidase positive.

## DISCUSSION

**Endemic nature of *Agrobacterium* spp.** *Agrobacterium* spp. are endemic to the Allison Savanna and appeared to be a natural component of the soil microbiota. Large populations of agrobacteria were detected in all soil and root samples examined. The population levels in the soil were higher than has been our experience for cultivated soils. This is the first

reported evidence for the presence of *Agrobacterium* spp. in undisturbed, nonagricultural soils, suggesting that this organism is not only a successful invader but also a true inhabitant of the midwest savanna soils. Furthermore, the data show that *Agrobacterium* spp. can reside on the roots of native plants, including an unidentified monocot within the family Poaceae.

**Predominance of nonpathogenic agrobacteria.** The inability of all 54 *Agrobacterium* strains to infect tomato seedlings, a relatively good indicator of pathogenicity (1), may be due to their host specificity (1). However, the majority of these strains are probably not pathogenic, because nonpathogenic agrobacteria largely predominate over pathogenic agrobacteria in cultivated soils and the rhizosphere of healthy host plants (30, 33) and can even prevail in crown gall tumors (4). This overwhelming predominance of nonpathogenic agrobacteria underscores the need to understand their role in the disease cycle. Are these nonpathogenic agrobacteria in a form which would have pathogenicity genes (oncogenicity, virulence, or host specificity genes) repressed until triggered by favorable environmental conditions, thus transforming this otherwise saprophyte into an active pathogen? Alternately,

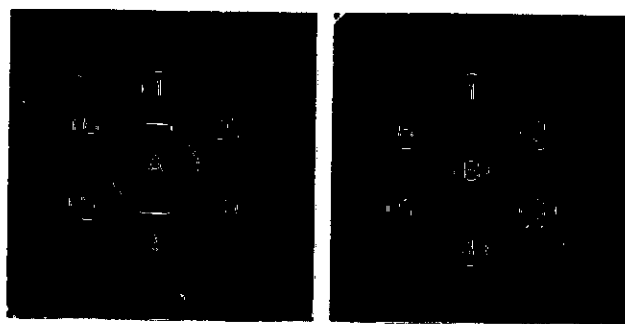


FIG. 1. Immunodiffusion patterns of ribosomal antisera to *Agrobacterium* spp. against strains isolated from the Allison Savanna. The center wells of panels A and B contain antisera, respectively, to *Agrobacterium* strain M63/79 and *Agrobacterium* strain U11. Outer wells of panel A contain ribosomes extracted from strains M63/79 (wells 1 and 4), R6/84 (group 1) (well 2), S2/84 (group 2) (well 3), S4/84 (group 4) (well 5), and R5/84 (group 1-2) (well 6). Outer wells of panel B contain ribosomes from strains U11 (wells 1 and 4), S4/84 (group 4) (well 2), R5/84 (group 1-2) (well 3), R6/84 (group 1) (well 5), and 3R25 (unidentified rhizosphere bacterium) (well 6).

TABLE 3. Number of strains using nopaline or octopine or both as the sole carbon and nitrogen source in solid or liquid media

Bacteria	Type of strains using			
	Nopaline only	Octopine only	Both	
	S <sup>a</sup>	L <sup>a</sup>	S	L
<i>Agrobacterium</i> physiological group 1-2			1	0
<i>Agrobacterium</i> physiological group 2			4	1
Nonfluorescent oxidase-positive	6	5	5	4
Nonfluorescent oxidase-negative	5	1	6	2

<sup>a</sup> These strains grew on either octopine or nopaline.

<sup>b</sup> Solid medium.

<sup>c</sup> Liquid medium.

tively, are these bacteria saprophytes until a pTi infects them?

Characteristics of naturally occurring agrobacteria. *Agrobacterium* biovar 2 strains predominated in this study and consisted of three phenotypically distinct groups. The largest group of biovar 2 strains isolated had the peculiar characteristic of not metabolizing erythritol, the carbon source used to selectively isolate biovar 2 agrobacteria (6, 29). The four biovar 1-2 strains differed from the representative biovar 3 strain CG64 in their utilization of erythritol and hence their growth on medium 2E. The 14 strains making up the fourth physiological group shared characteristics of both biovars 2 and 3. Members of this group did not grow on medium 3DG and differed from biovar 3 in their oxidase reaction, their ability to use erythritol, and their inability to utilize L-(+)-tartaric acid; they differed from biovar 2 in their inability to utilize L-tyrosine, mucic acid, L-(+)-tartaric acid, and malonate.

A problem confronted in this study, as well as in that of Spiess et al. (36), is how to identify nonpathogenic agrobacteria which do not fit in the accepted biovar groupings (16). However, we were able to identify the savanna strains to the genus level by using antisera to 50S ribosomal subunits of *Agrobacterium* spp. and our limited nodulation assays.

The existence of new physiological groups should not be surprising, because reports of pathogenic *Agrobacterium* strains that do not fit the assigned biovar are common (2, 10, 13, 26, 37; A. R. Anderson, Ph.D. thesis, Oregon State University, Corvallis, 1978). Future searches for *Agrobacterium* spp. in new habitats outside nurseries and orchards will probably demonstrate the ubiquitous nature of this group of organisms, their adaptation to different environments, and the presence of new biovars. Care should be taken not to rely solely on selective media in this search, because the new biovars may not grow on these media.

Preponderance of nonagrobacteria among opine utilizers. Opines, which are chemicals synthesized specifically by plant tissue genetically transformed by the pTi of *Agrobacterium* spp., are described as mediators of parasitism in the interactions between the pTi, its *Agrobacterium* host, and the tumorigenic plant cells (38). In this context, the bacterium harboring a pTi induces the development of a crown gall tumor, which is a specific ecological niche for itself, rich in the opine that only it can metabolize, favoring its growth and propagation (11). However, it has now been established that opine utilization is not confined to pathogenic agrobacteria. Strains of nonpathogenic agrobacteria (18, 24, 25, 27) and fluorescent pseudomonads (2, 6, 24, 31; L. W. Moore, Fourth Int. Congress Phytopathol. abstr. no. 141, 1983) were reported to utilize opines. In the present study, 30 of 126 strains isolated from soil and root samples grew on solid medium containing opines as the sole carbon and nitrogen source, but only 12 utilized opines in liquid medium. This demonstrates that the use of solid medium should be limited to rapid screening of opine utilizers. The fact that 83% of the opine utilizers were neither agrobacteria nor fluorescent pseudomonads clearly indicates that a large and diverse microbiota can utilize opines. Apparently, a pathogenic *Agrobacterium* strain faces a stronger competition in the opine environment it induced than was previously thought. The present isolation of opine utilizers from an environment a priori free of crown gall must lead us to rethink the origin and role of opines in the terrestrial environment and determine the ecological significance of opine utilization by bacteria which are not agrobacteria.

#### ACKNOWLEDGMENTS

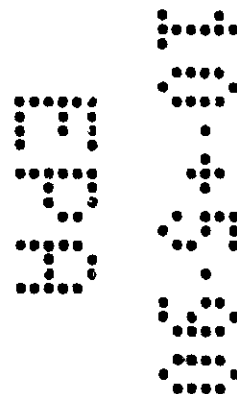
This work was supported in part by the Ministère de l'Enseignement Supérieur of Algeria.

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CROWN GALL OF STONE FRUIT

I. ISOLATION OF *AGROBACTERIUM TUMEFACIENS* AND RELATED SPECIES

By A. KERR\*

[Manuscript received June 17, 1968]

Summary

Two distinct forms of tumour-inducing bacteria have been isolated. One form, readily isolated when macerated galls were plated on Patel's medium, did not grow on Schroth's medium even when antibiotics and other bacterial inhibitors were omitted; nor did it produce 3-ketolactose when grown on lactose agar. It is considered to be the primary cause of crown gall in at least one commercial nursery.

The second form was rarely isolated from galls or from soil surrounding galls. It grew on Schroth's medium and produced 3-ketolactose, and was designated *Agrobacterium tumefaciens*. Its role in the etiology of crown gall is doubtful. *A. radiobacter* inhibited gall induction by *A. tumefaciens*, and this is likely to operate in the field, where the ratio of the two species is more than 100:1.

I. INTRODUCTION

The nature of crown gall was established by Smith and Townsend (1907) who showed that the disease is caused by a bacterium which they named *Bacterium tumefaciens*, later changed by Conn (1942) to *Agrobacterium tumefaciens*.

In South Australia, crown gall is widespread and serious, particularly on peach, almond, and apricot. It presents considerable problems in nurseries, where disease incidence is often high but varies markedly from year to year. Although only plants without galls are sold by a nursery, the possibility of transporting the causal organism in soil attached to the roots is very real. Yet little is known about the ecology of *A. tumefaciens* or its distribution and abundance in nurseries and orchards, either in South Australia or elsewhere. The main reason for this is the difficulty of distinguishing *A. tumefaciens* from other soil-inhabiting bacteria. Recently, Schroth, Thompson, and Hildebrand (1965) described a selective medium for the *A. tumefaciens*-*A. radiobacter* system, allowing quantitative assessment of these bacteria in soil. Where the identity of a particular isolate, this can be dispelled by a variety of tests (Bernaerts and De Ley 1963).

It is hoped that two techniques would allow a much more detailed study of the ecology and distribution of *A. tumefaciens* than had previously been possible. This paper describes preliminary investigations largely directed towards assessing the suitability of the selective medium described by Schroth, Thompson and Hildebrand (1965) for isolating *A. tumefaciens* from soil and from galls.

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## II. METHODS

(a) Isolation of *Agrobacterium* spp. from Soil

The soil dilution plate method was used: 0.1 ml of an appropriate soil dilution (usually 1:10 or 1:50) was pipetted on to Schroth's medium in a 9-cm Petri dish and spread evenly over the surface with a sterile L-shaped glass rod. The plates were incubated at 27°C for 3 days and then selected individual bacterial colonies were transferred to nutrient agar slopes.

(b) Isolation of *Agrobacterium* spp. from Galls

Galls or parts of galls showing no sign of secondary rotting were removed from naturally infected plants and thoroughly washed in tap water; 0.5–1.0 g of tissue was surface-sterilized in 0.5% sodium hypochlorite for 10 min, washed briefly, and then macerated in a pestle and mortar with 10 ml of sterile distilled water. The macerate was transferred to a sterile Potter-Elvehjem homogenizer and the pestle depressed, giving a supernatant suspension free from large particles. The supernatant was diluted (1:100) with sterile distilled water and plated on Schroth's medium, or, in the later stages of the investigation, on Patel's medium (Patel 1926).

A similar method was used for re-isolation of bacteria from artificially inoculated plants, except that a 3.0-cm length of stem, which included the site of inoculation, was removed and macerated in an electric blender before transferring to a homogenizer.

In both cases, selected colonies were transferred to nutrient agar slopes.

## (c) Identification and Pathogenicity of Isolates

All isolates were tested for 3-ketolactose production (Bernaerts and De Ley 1963), a quick and specific test for the *A. tumefaciens*-*A. radiobacter* group of bacteria. To distinguish between *A. tumefaciens* and *A. radiobacter* it is necessary to determine pathogenicity. Isolates were grown on nutrient agar slopes at 27°C for 48 hr. A heavy suspension of bacterial cells was prepared and deposited on young tomato plant stems which were then wounded by an instrument similar to that described by Huisingh and Durbin (1968) but with no cotton wool. Inoculated plants were kept at a temperature of 27°C for 3 days and were then transferred to a glasshouse. Results were recorded after 10 days. Young peach seedlings were inoculated in the same way and also by wounding roots before applying a bacterial suspension. If isolates produced 3-ketolactose, pathogens were designated *A. tumefaciens* and non-pathogens *A. radiobacter*.

## III. RESULTS

## (a) Isolations from Soils and Galls

Eighteen soil samples were collected from the sites of crown gall-infected stone fruit trees in several localities, and 36 samples from various other sites, where no crown gall-infected trees were observed. From infested sites, only one isolate out of 195 tested was pathogenic. No pathogens were isolated from other sites (Table 1).

Because of the infrequent isolation of *A. tumefaciens* from soil, isolations from galls from six different localities were attempted using the same selective medium. From 33 galls, 385 isolates, all ketolactose-positive, were tested for pathogenicity; only five were pathogenic (Table 1).

(b) Inoculation of Tomato Plants with *A. tumefaciens* and *A. radiobacter*

Since differentiation of species is based on pathogenicity, a loss of virulence following infection could explain the difficulty of isolating *A. tumefaciens* from natural galls. Alternatively *A. tumefaciens* could be swamped by *A. radiobacter* after tumour induction. These possibilities were investigated. Tomato plants were

inoculated with *A. tumefaciens* and *A. radiobacter* both separately and mixed in the ratio of 1:3. Galls developed following inoculation with *A. tumefaciens* and with the mixture. Bacteria were isolated from inoculated plants over a period of 8 weeks using three plants per treatment. On each occasion 30 isolates were selected at random from each treatment and tested for pathogenicity. Results are given in

TABLE 1  
FREQUENCY OF RECOVERY OF PATHOGENIC ISOLATES FROM GALLS AND  
FROM SOIL PLATED ON SCHROTH'S MEDIUM

	Source		
	Galls	Soil Surrounding Galls	Other Soils
No. of samples	33	18	36
No. of isolates	385	195	88
No. of pathogenic isolates	5	1	0
No. of samples yielding pathogenic isolates	2	1	0

TABLE 2  
NUMBER (OUT OF 30) OF PATHOGENIC ISOLATES FROM TOMATO STEMS  
INOCULATED WITH *A. TUMEFACIENS*, *A. RADIOBACTER*, OR A  
MIXTURE OF BOTH (IN THE RATIO 1:3)

	No. of Pathogenic Isolates at Various Weeks after Inoculation:								Total Pathogenic Isolates
	0	1	2	3	4	5	6	8	
Inoculated with:									
<i>A. tumefaciens</i>	29	30	30	28	29	25	28	30	229
1/3 Mixture	3 $\frac{1}{10}$	7 $\frac{1}{4}$	6 $\frac{1}{5}$	6 $\frac{1}{5}$	9 $\frac{1}{3}$	9 $\frac{1}{3}$	9 $\frac{1}{3}$	7 $\frac{1}{4}$	56
<i>A. radiobacter</i>	0	0	0	0	0	0	0	0	0

Table 2. Of 240 isolates tested for each treatment, plants inoculated with *A. tumefaciens* yielded 229 pathogens, those inoculated with the mixture, 56 pathogens, and those inoculated with *A. radiobacter*, none. It can be concluded that a marked change in pathogenicity is unlikely to occur following inoculation and that *A. tumefaciens* is not swamped by *A. radiobacter* after tumour induction.

Lippincott and Lippincott (1967) have reported that the presence of non-pathogenic isolates of *A. tumefaciens* restricts tumour induction by pathogens, presumably by competing for infection sites. As the ratio of pathogens to non-patho-

gens in infested soil appears to be less than 1:100, the possibility of interference was investigated. Bacterial suspensions of *A. tumefaciens* and *A. radiobacter* were mixed to give a ratio of 1:100 and then serially diluted. The pathogenicity of the mixture was compared with that of a pure culture of *A. tumefaciens* also serially diluted. Numbers of bacteria were determined by plating before inoculation. Young tomato plants were wounded in four places with a blunt needle and 0.05 ml of bacterial suspension deposited on each wound. Stem diameters at inoculation sites were measured after 5 weeks, and the results are shown in the following tabulation:

No. of <i>A. tumefaciens</i> cells per wound	$12.5 \times 10^4$	$12.5 \times 10^3$	$12.5 \times 10^2$	12.5	0
Mean diam. (cm)* of tomato stems following inoculation with:					
<i>A. tumefaciens</i>	1.61 <sup>1</sup>	1.39 <sup>2</sup>	1.08 <sup>3</sup>	0.79 <sup>4</sup>	0.65 <sup>5</sup>
Mixture†	—	0.83 <sup>4</sup>	0.69 <sup>5</sup>	0.64 <sup>5</sup>	0.65 <sup>5</sup>

\* Means with different superscript numbers are significantly different at the 1% level.  
† *A. tumefaciens* and *A. radiobacter* in the ratio 1:100.

The presence of *A. radiobacter* markedly restricted or completely inhibited tumour development, depending on the number of *A. tumefaciens* cells per wound. These results confirm those of Lippincott and Lippincott (1967) who showed that the inhibition is fairly specific; unrelated bacteria and some strains of *Agrobacterium* failed to inhibit. This might explain why Beaud, Manigault, and Stoll (1963) found no evidence of inhibition.

#### (c) Pathogenicity of Isolates to Peach and Tomato

These results cast doubt on the value of Schroth's medium for isolating *A. tumefaciens* both from soil and from galls though there is the further possibility that isolates from stone fruit may not be pathogenic to tomato. Six pathogenic and 100 non-pathogenic isolates, obtained during the present investigation, as well as 12 pathogenic isolates supplied from other laboratories within Australia, were inoculated into both tomato and peach plants. All isolates pathogenic to tomato induced crown gall development on peach stems and roots; all other isolates were non-pathogenic to both tomato and peach.

#### (d) Pathogenicity of Bacteria Isolated on Schroth's and Patel's Media

It would seem that the most likely explanation for the infrequent isolation of tumour-inducing bacteria from galls, or from soil surrounding galls, is that the medium described by Schroth, Thompson, and Hildebrand (1965) is not suitable. This was investigated by plating galls on both Schroth's and Patel's media and determining the pathogenicity of isolates. Young galls were collected on three separate occasions from peach and plum seedlings in a commercial nursery, macerated, diluted 1:1000, and plated on both media. Isolates were obtained from individual colonies on each medium and tested for 3-ketolactose production and for pathogenicity to tomato (Table 3). Of 112 isolates from Schroth's medium, none were pathogenic; although all produced 3-ketolactose. Of the 117 isolates from Patel's medium, 85 were patho-

genic. Of the non-pathogens, approximately half produced 3-ketolactose and can be designated *A. radiobacter*; the remainder have been identified as *Enterobacter* sp. (Hayward, private communication) and were readily distinguishable from the pathogens by cultural appearance. The inability of the pathogenic isolates to grow on Schroth's medium was confirmed by plating the bacteria on this medium, when no growth was observed. Nor will they grow on Schroth's basal medium which lacks antibiotics and other bacterial inhibitors. These isolates are tentatively designated *Agrobacterium* sp. and six were tested for pathogenicity to peach. All induced crown gall development.

TABLE 3  
3-KETOLACTOSE PRODUCTION AND PATHOGENICITY OF BACTERIAL  
ISOLATES FROM GALLS PLATED ON SCHROTH'S AND PATEL'S MEDIA

No. of Isolates	Isolation Medium	3-Ketolactose	Pathogenicity
112	Schroth	—	—
17	Patel	—	—
85	Patel	—	+
15	Patel	—	—

#### IV. DISCUSSION

Crown galls collected from one nursery were plated on both Schroth's and Patel's media. As tumour-inducing bacteria were isolated only on Patel's medium, it seems clear that Schroth's medium is not suitable for the isolation of these bacteria. That this applies to samples from other areas is suggested by the very infrequent isolation on this medium of pathogenic bacteria from 33 galls taken from six different localities.

Nomenclature of the isolates deserves comment. Pathogenic isolates were designated *A. tumefaciens* and non-pathogens *A. radiobacter* if they could grow on Schroth's medium and produce 3-ketolactose when grown on lactose agar. This is a very arbitrary identification but is nevertheless very convenient for distinguishing these isolates from others, tentatively designated *Agrobacterium* sp., which can induce crown gall but cannot produce 3-ketolactose or grow on Schroth's medium even when antibiotics and other bacterial inhibitors are omitted. Final identification awaits detailed taxonomic study, but all three types would be included in phenon I of Thornley (1967) along with other agrobacteria (Hayward, private communication). It is relevant that De Ley *et al.* (1966) found that 4 of 28 strains of *A. tumefaciens* did not produce 3-ketolactose.

Both *A. tumefaciens* and the *Agrobacterium* sp. produce identical symptoms when inoculated into tomato or peach. The frequent isolation of *Agrobacterium* sp. would suggest that it is the primary cause of crown gall in at least one nursery.

The role of *A. tumefaciens* is puzzling. It is rarely isolated from galls and this cannot be explained by a loss of virulence following infection, because when plants were inoculated, more than 95% of the re-isolates obtained over a period of 8 weeks were pathogenic. Nor was *A. tumefaciens* swamped by *A. radiobacter* when a mixture of the two bacteria in the ratio 1:3 was inoculated into tomato. The ratio of the two organisms before, and for 8 weeks after, inoculation remained relatively constant. The evidence indicates that the infrequent isolation of *A. tumefaciens* from natural samples reflects its low numbers in the field.

In soil, the ratio of *A. tumefaciens* to *A. radiobacter* is less than 1:100, but when these organisms are mixed in this ratio and then inoculated into tomato stems, gall formation is either completely inhibited or markedly reduced compared with that induced by the same number of *A. tumefaciens* cells in pure culture. In other words, *A. radiobacter* inhibits gall induction by *A. tumefaciens*. As soil is the only source of inoculum for natural infection of plants, these results suggest that *A. tumefaciens* does not cause natural infection. Nevertheless *A. tumefaciens* was isolated from two galls. It is possible that there are local pockets in the soil where the ratio of *A. tumefaciens* to *A. radiobacter* is greater than 1:100. Another possible explanation is that there is a transfer of virulence from the *Agrobacterium* sp. to *A. radiobacter*, changing the latter into *A. tumefaciens*. If this were a relatively rare occurrence, it could explain the low numbers of *A. tumefaciens* in the field.

#### V. ACKNOWLEDGMENTS

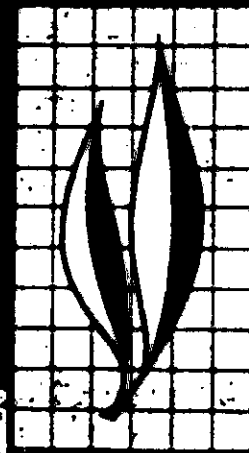
I wish to thank Dr. A. C. Hayward for preliminary taxonomic studies on several of the isolates, Dr. Rose Mushin and Dr. C. A. Parker for supplying bacterial cultures, and Miss E. Farrow for technical assistance.

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# HILGARDIA

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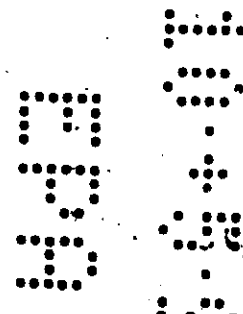


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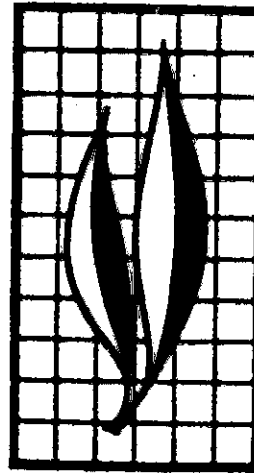
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## Biology and Control of *Agrobacterium tumefaciens*

M. N. Schroth, A. R. Weinhold, A. H. McCain,  
D. C. Hildebrand, and N. Ross



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This paper presents recent information on the biology of *Agrobacterium tumefaciens*, and outlines techniques for isolating and studying the bacterium. Data are presented indicating that *A. tumefaciens* is a soil inhabitant. The organism was isolated in 18 of 28 California soils, including soils not known to support host plants, and appears to be a rhizosphere organism. *In vitro* experiments showed that the bacterial cells aggregated around roots of various plants.

*A. tumefaciens* and *A. radiobacter* were studied for serological and physiological differences. Although no physiological differences were detected, most *A. tumefaciens* strains differed serologically from *A. radiobacter* in possessing an antigen which formed a precipitin line near the antigen well in gel-diffusion tests. The last part of the paper discusses how the disease is contracted and presents techniques for reducing loss from crown gall.

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M. N. Schroth, A. R. Weinhold,  
A. H. McCain, D. C. Hildebrand,  
and N. Ross

## Biology and Control of *Agrobacterium tumefaciens*\*

### INTRODUCTION

THE BACTERIUM *Agrobacterium tumefaciens* has an ubiquitous host range among dicotyledonous plants and causes disease in many parts of the world. Disease incidence has steadily increased in many California nurseries and orchards along with their increase in production. In 1968 crown gall was judged one of the three most important diseases on 14 major crops in California, causing an annual loss of \$7 million per year (University of California Plant Pathology Statewide Conference on Plant Disease Losses Committee, 1965).

Although *A. tumefaciens* has been intensively studied for over 60 years, little data exist concerning its biology in soil. It is not known how long the pathogen survives, what factors favor its multi-

plication, whether it is a rhizosphere organism, and what the best methods are to eradicate it from soil. In response to the request of California growers for assistance in controlling the disease, an investigation was initiated to study the biology of the causal organism and to develop economically feasible control methods.

Some of the information resulting from these studies has already been published (Ross *et al.* 1970). Although there still are no specific answers to all of the questions mentioned, this report contains the latest information on the biology of the organism and describes our techniques for studying the organism and reducing loss from crown gall disease. Table 1 lists the organisms used in the study.

### ISOLATING THE ORGANISM

Efficient methods for isolation and rapid identification of the *A. tumefaciens* are essential for studying its ecology in soil and the lack of such methods is largely responsible for the scanty attention given to this aspect of the disease cycle. Therefore, we developed two methods for selectively isolating agrobacteria from soil. The first method consists of placing soil directly on fresh car-

rot slices and incubating them for 10 to 15 days (Ark and Schroth, 1958). The development of galls indicates that *A. tumefaciens* is present in the soil. However, this method does not provide quantitative data about the population in the soil and has the further disadvantage that carrot slices are often rotted by other bacteria.

The second isolation procedure con-

\* Submitted for publication April 30, 1970.

TABLE 1  
ORIGINS OF ISOLATES USED IN THE STUDY

Genus and isolate number	Isolated from	Source
<i>Agrobacterium radiobacter</i>		
Rad-1	Soil	M. N. Schroth
Rad-2	Soil	M. N. Schroth
Rad-6466	Soil	American Type Culture Collection
Rad-6467	Soil	American Type Culture Collection
Rad-B	Soil	M. N. Schroth
Rad-C	Soil	M. N. Schroth
Rad-D	Soil	M. N. Schroth
TR-1	Soil	M. P. Starr, ICPPB
TR-4	Soil	M. P. Starr, ICPPB
TR-4	Soil	M. P. Starr, ICPPB
TR-102	Soil	M. P. Starr, ICPPB
TR-106	Soil	M. P. Starr, ICPPB
<i>A. rhizogenes</i>		
Rhi-1	Rose	P. A. Ark
Rhi-2	Rose	P. A. Ark
<i>A. tumefaciens</i>		
Ach-1	Achilles	P. A. Ark
Al-1	Almond	M. N. Schroth
Al-2	Almond	E. Volcani
Ap-1*	Apple	M. N. Schroth
Apr-1	Apricot	M. N. Schroth
Apr-A	Apricot	M. N. Schroth
Apr-B	Apricot	M. N. Schroth
B-32	Bryophyllum	P. A. Ark
B-48	Bryophyllum	P. A. Ark
B-55	Bryophyllum	P. A. Ark
Ced-1*	Incense cedar	M. N. Schroth
CG-1	Pear	P. A. Ark
Dah-1	Dahlia	E. Volcani
Ded-1	Dedounea	M. N. Schroth
Eu-2	Eucalyptus	P. A. Ark
Fa-1	Fusch	M. N. Schroth
Fl-1	Flum	M. N. Schroth
Pop-1*	Poppy	M. N. Schroth
R-12	Hollyhock	Unknown
Rub-1	Rubbery	M. N. Schroth
S Bak-1	Soil	M. N. Schroth
Sba-1	Sugar beet	J. P. Thompson
TT-2		M. P. Starr, ICPPB
<i>Rhizobium japonicum</i>		
RJ-1	Soybean	Shirley Nash Smith
<i>R. meliloti</i>		
Rm-1	Sweet clover	Shirley Nash Smith

\* Although these strains were isolated from tumors, they were not virulent and could therefore also be considered to be *A. radiobacter*.

sisted of plating soil on a selective medium (Schroth et al., 1965) composed of a combination of antibiotics and nutrients that exclude over 99 per cent of the microorganisms which develop on standard media. *A. tumefaciens* and *A. radiobacter* colonies are easily distinguished from other bacterial colonies by their characteristic appearance. The medium has been useful in assaying soils for populations of *A. tumefaciens* and

*A. radiobacter*, and its plating efficiency is indicated by the fact that average recovery of cells of different isolates introduced into soil was 38 per cent. However, certain strains of *A. tumefaciens* do not grow on it. Thirty-nine strains of *A. tumefaciens* were compared for growth on this medium and on potato-dextrose-peptone agar (PDP). Two tenths ml of a  $10^6$  cell per ml of suspension was pipetted onto the surfaces of

Isola

Apr A.  
Apr B.  
CG 1.  
Cg Alm  
Ded 1.  
Eu 7.  
S. Bak  
SBA 1.  
T 2

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TABLE 2  
NUMBER OF COLONIES OF *A. TUMEFACIENS* FORMED ON VARIOUS MEDIA WITHOUT SOIL SOLUTION\*

Isolate number	Medium			
	Nutrient agar	Patel's	PDP	Schroth's
	Number of colonies			
Apr A.....	12,300	10,000	...	8
Apr B.....	1,300	1,300	400	3
CG 1.....	6,800	5,500	300	1
Cg Alm.....	7,000	2,300	1,100	41
Dad 1.....	44	30	30	4
Da 7.....	1,215	900	670	140
S. Bak 1.....	30	30	30	1
SBA 1.....	5,700	1,000	2,700	0
T 2.....	11,200	10,400	7,000	4

\* Each figure is the mean of 5 replications and represents number of colonies formed per ml of bacterial suspension on different media. Suspensions were standardized, using a Klett reading of 50.

the media and distributed with an L-shaped glass rod. Only 17 of the 39 strains grew on the selective medium, and, surprisingly, only 23 grew on PDP. Kerr (1969) reports that some strains occurring in Australia do not grow on the selective medium. This is probably because of the Australian isolates' inability to use nitrate; we have found that these strains will grow if ammonium salts are added to the medium as the nitrogen source.

Although the selective medium is effective for isolation purposes, it is not a good medium for culturing the organism. The ability of nine strains to produce single colonies in Patel's medium (Patel, 1926), PDP, nutrient agar, and the selective medium without soil solution, was evaluated by plating a dilution series on these media. The greatest number of colonies developed on nutrient agar, and the least on the selective medium (table 2). The plating recovery of cells introduced into soil was 38 per cent, but the average number of colonies formed on the selective medium without soil solution averaged only 0.61 per cent, as compared to that on nutrient agar.

It is not surprising that the selective medium operates effectively as an isolation medium when a soil suspension is

used, as the medium was developed by plating soil solutions on it, recording the results, and then adjusting the balance of nutrients and antibiotics. The selective medium probably lacks compounds required for bacterial growth but which are present in the soil solution. Thus the medium is essentially a basal medium until soil solution is added.

While developing the selective me-

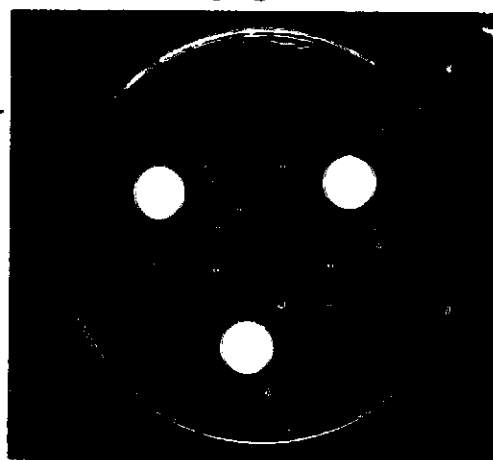


Fig. 1. Growth of *Agrobacterium tumefaciens*, isolate R-12, on potato-dextrose-peptone agar, demonstrating that culture filtrates provide required substance(s) not present in the medium. Surface of 30° plate was seeded with bacteria at a concentration of 10<sup>6</sup> cells per ml. The discs shown in this photograph contain cultural filtrate from a nutrient broth culture of R-12 grown for 2 days.

Did this work?

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dium it was noted that some strains did not grow unless a heavy suspension or a mass transfer of cells was applied to the medium. This also occurred with other media such as PDP and nutrient agar. The problem was solved by first incorporating sterile culture filtrate into these media. Striking results were obtained by soaking filter paper discs in bacterial filtrate and placing them on a PDP dish previously seeded with the filtrate requiring strain at a cell count

of  $10^6$  per ml (fig. 1). Growth was considerably greater about the discs, and in some cases (as with strain R-12) growth occurred only around the discs. This suggests that data collected from plate counts should be evaluated precisely, as poor nutrition or lack of growth requirements may result in highly inaccurate counts. Colonies often may develop only if there is an aggregate of cells present, and not from a single cell.

## IDENTIFICATION OF *A. TUMEFACIENS*

The Bernaerts-De Ley test (Bernaerts and De Ley, 1963) for 3-ketoglycoside-formation is a simple, rapid test for general identification of the *A. tumefaciens-radiobacter* group. The test is performed by spotting suspected *Agrobacterium* (4 to 6 spots per plate) on the Bernaerts-De Ley medium (0.1 per cent yeast extract, 1 per cent lactose, and 2 per cent agar). The plates are incubated at 28°C. for 1 to 2 days, then flooded with Benedict's reducing sugar reagent at room temperature. The formation of a yellow ring around the colony after 15 minutes to 1 hour is indicative of both *A. tumefaciens* and *A. radiobacter*. However, we have several strains which

do not form these substances. Similar non-3-ketoglycoside-forming strains have been reported from Australia (Kerr, 1969).

At present there is no rapid method for distinguishing between *A. tumefaciens* and *A. radiobacter*, although serology shows promise for doing this. The best present method is to inoculate a host plant. This is most easily accomplished by inoculating either carrot slices, young tomato, or sunflower plants; tumors will appear approximately 7 to 10 days after inoculation if the bacterium is *A. tumefaciens*. Care must be taken not to confuse wound callus tissue with gall tissue.

## SURVIVAL OF *A. TUMEFACIENS* IN SOIL

The ability of *A. tumefaciens* to survive in soil for long periods is controversial. Reports that the organism is not a good soil inhabitant (Hildebrand, 1941) may result from lack of adequate techniques for isolating the bacteria. The ability of the organism to survive is an important facet of the disease cycle because it affects the type of control procedure to apply (such as crop rotation, soil fallow, fumigation, or movement of a nursery to an area known not to have supported host plants).

Isolation of *A. tumefaciens* and *A. ra-*

*liobacter* on the selective medium discussed above indicated that both organisms are widespread in California soils. *A. tumefaciens* was detected in 18 of 28 soils tested. Only one of 28 California soils tested failed to yield *A. radiobacter* when plated directly on the selective medium—but the organism was recovered even from this soil when the selective medium was used as an enrichment medium. For this purpose, the selective medium was prepared without agar, 1 gram of soil was added to 100 ml of the broth, and the mixture was incu-

**TABLE 3**  
**POPULATION OF *A. TUMEFACIENS* AND *A. RADIOBACTER* IN SOIL AS  
DETERMINED BY PLATING ON THE CROWN GALL SELECTIVE MEDIUM**

Area and soil type*	Crop	Population per gram of soil†	
		<i>A. tumefaciens</i>	<i>A. radiobacter</i>
Bakersfield—sandy loam.....	Cotton field	30	9,120
sand.....	Woods	5	600
Butte County—sandy loam			
Before fumigation.....	Nursery	272	3,400
After fumigation.....	Nursery	77	900
Madera—sandy loam.....	Almond	180	2,300
Pittsburg—sandy loam.....	Almond	48	5,000
Salisbury—clay loam.....	Bean	5	2,300

\* The Behrensfield soils were from a cotton field and an area near a river; the Pittsburg soil was from a walnut orchard; the Sullivan soil was from a grass pasture; and the other soils were from nurseries where stone fruits had been planted.

bated for 48 hours after which two-tenths of a ml was plated on the standard selective medium. Table 3 shows that the ratio of *A. tumefaciens* to *A. radiobacter* in seven of the eighteen soils harboring both species varied from 1:13 to 1:500.

The lowest ratio occurred in nursery soils where stone fruits had been planted. The grass pasture (Salinas soil, table 3) had, supposedly, never been cultivated and had not supported host plants other than dicotyledonous weeds. We have also isolated *A. tumefaciens* from three other fields not known to have grown host plants other than weeds. *A. tumefaciens* was also occasionally isolated from soils of cotton and tomato fields and from fields left fallow for over 5 years, using the carrot technique. Greatest success occurred when pieces of root material with clinging soil were placed on carrot slices.

Additional studies on populations of *A. tumefaciens* in soils have not been conducted because of the inability to readily distinguish between virulent and avirulent strains. However, certain strains of *A. tumefaciens* can easily be distinguished from other strains on the

selective medium because of a particular colony appearance, such as a deep-red color. These strains could be used to study population dynamics in soil.

The above findings indicate that in many areas of California *A. tumefaciens* is either a soil inhabitant or is able to persist for years after introduction. Survival most likely is affected by multiplication in the rhizospheres of plants and occasional increase by infection of susceptible weeds and commercial crops. There is no assurance, therefore, that planting of nursery trees in soil not known to have supported economic host plants will result in the production of disease-free trees. The ability of *A. tumefaciens* to survive in the soil is not unexpected because *A. radiobacter*, which is the same as *A. tumefaciens* except for virulence (De Ley, 1968), has long been considered a normal rhizosphere bacterium by microbiologists. Dickey (1961) reported that *A. tumefaciens* survived for long periods in fertilized and unfertilized soil contained in tumblers without the presence of a host, and in view of this he concluded that the organism must be considered a successful soil invader.

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## ATTRACTION OF AGROBACTERIA TO ROOTS

*A. tumefaciens* commonly attacks the crown and roots of plants. This and the observations that members of *Rhizobium*, a genus related to *Agrobacterium*, often aggregate around roots (Fahraeus, 1957; Thorton, 1936) prompted an investigation to ascertain if *A. tumefaciens* also might be attracted to roots. An abstract on this study has been published (Schroth and Ting, 1966).

Seeds of pea, mung bean, tomato, tobacco, barley, and cucumber were sterilized in a 0.5 per cent aqueous solution of sodium hypochlorite for 3 minutes and then germinated on moist filter paper in petri dishes. Excised root and intact seedlings were placed in drops of water containing bacteria, placed on microscope slides, and covered with a cover slip. Movement of bacteria was observed by phase microscopy at 400x.

Bacteria of most strains were observed to cling to root hairs and zones of elongation of roots on all plants examined (fig. 2, A, B). Strands of bacteria were often observed between two root hairs, as if there was an attraction be-

tween them (fig. 2A). Only 13 of 46 strains of *A. tumefaciens* failed to accumulate around root hairs. Generally, the accumulation of bacteria around the hairs was slow, but bacteria often could be seen darting to a specific site on a root hair, oscillating for one to several seconds, then departing in another direction. This specific site would be visited by a number of bacteria. Broken root hairs also appeared to be major sites for bacteria accumulation. There was a distinct zone of repulsion around the root-cap zone of all plants.

It is tempting to speculate that bacterial accumulation in roots and wounds may have an important role in the initiation of disease. However, it is also possible that the phenomenon may not operate in the rhizosphere because of the effect of other microflora and charged soil particles. Although this phenomenon has been observed hundreds of times, attraction of agrobacteria to root hairs does not always occur, often we make several tests before attraction occurs. Attempts to determine the source of variability have failed.

## SEROLOGY OF AGROBACTERIUM

Many techniques have been unsuccessfully used in attempts to easily distinguish *A. tumefaciens* from *A. radiobacter*. The principle characteristic distinguishing *A. tumefaciens* from *A. radiobacter* is its ability to cause tumors. However, Hoehstet and Cole (1967) investigated the serological characteristics of four virulent and four avirulent strains of *A. tumefaciens* and concluded that serology probably could be used to identify strains of the organism. An investigation was therefore made of the serological properties of *A. radiobacter* and *A. tumefaciens*, as this would greatly assist a study of the population dynamics in soil since it is necessary to

distinguish between virulent and avirulent strains. *A. rhizogenes* and *Rhizobium* species were included in the investigation for comparative purposes.

The 12 virulent strains of *A. tumefaciens* used in the study were from plum (Pl-1), almond (Al-2), peach (Pe-1), dahlia (Dah-1), pear (CG-1), apricot (Apr-1), and bryophyllum (B-38, B-48, and B-55). The four avirulent isolates tested were from apple (Ap-1), poppy (Pop-1), cedar (Ced-1), and strain Ach-1 (which became avirulent in culture) from *Achillea*. These strains, since they are avirulent technically, probably should be called *A. radiobacter*. Because strain Ach-1 initi-



Fig. 2, A and B. Accumulation of *A. tumefaciens* cells around the root hairs of a tomato plant. A also shows a line of bacteria as commonly observed between two hairs.

ally was examined for purity only by streaking several times on a medium, it is possible that the culture was a mixture of *A. tumefaciens* and *A. radiobacter*.

Other organisms tested were ten strains of *A. radiobacter* (table 1), *Rhizobium japonicum*, and *R. meliloti*, and two isolates of *A. rhisogenes*.

Antisera for *A. tumefaciens* strains (CG-1, B-38, B-55, Al-2, and Eu-2) were prepared from rabbits injected with acetone-treated bacterial cells and whole cells, using techniques described by Kabat and Mayer (1961). Serological relations were determined by means of the Ouchterlony (1958) double-gel diffusion test. Titers of the antisera after concentration with  $(\text{NH}_4)_2\text{SO}_4$  were between 3,000 and 5,000.

Results from challenging the antigens from *A. tumefaciens* and *A. radiobacter* strains against antisera of *A. tumefaciens* suggested that *A. tumefaciens* possessed a distinctive antigen. Four to five lines with one dominant line *a* being formed near the antiserum well and another *b* near the antigen well, consist-

ently occurred when challenging *A. tumefaciens* antigen against antisera of *A. tumefaciens*. Some of the lines were broad and could be divided into additional lines, using different techniques. The two exceptions to this serological pattern were with strain B-38 (rose) and Dah-1 (dahlia), as a *b* line was not formed next to the antigen well when challenged against antiserum from other strains. However, antigen of B-38 produced line *b* when reacted against its homologous antiserum. Antiserum was not prepared for Dah-1; accordingly, the antigen of CG-1 did not produce line *b* when challenged against antiserum of B-38.

The principle difference in the serological pattern between *A. radiobacter* strains, or avirulent strains, and virulent *A. tumefaciens* strains, was that the antigen from none of the avirulent strains produced line *b* when challenged against *A. tumefaciens* antisera. The one exception to this observation was strain Ap-1 (apple) which was isolated from a tumor but which did not produce galls on test plants.



Antigen from *R. meliloti* reacted with antisera of *A. tumefaciens* and produced one precipitin line next to the antiserum well and the other in the middle between the wells. A positive agglutination reaction was previously reported by Graham (1963). *R. japonicum* did not produce a reaction.

Gel-diffusion tests indicated that *A. rhizogenes* was not serologically related to *A. tumefaciens*, since precipitin lines were not formed. Attempts were made to separate the various antigens with Millipore filtration, and with differential extraction using physiological saline. The degree of separation of the antigens was tested by the double gel-diffusion technique previously used. Cells were grown in nutrient broth plus 1.0 per cent glucose, centrifuged, and washed three times with saline. The antigen extracted with each washing was precipitated by acetone and then tested. The separation of antigen was affected by pressure filtration through a 0.22- $\mu$  Millipore filter.

Both the saline and Millipore filtration technique gave some separation of antigens. Although the first washing of cells with saline contained portions of all the antigens, the second and third washings contained mostly antigen *b*. This suggests that perhaps this antigen is more closely associated with the cell

wall than antigens easily removed. With Millipore filtration, antigens *a* and the middle ones passed through the filter, whereas antigen *b* was mostly retained.

Because of two exceptions to the findings that precipitin line *b* is specific to *A. tumefaciens*, results are inconclusive as to whether or not the virulent *A. tumefaciens* strain can consistently be serologically separated from avirulent strains and more work should be done to ascertain if the exceptions are authentic. Possibly, strain Ap-1 might produce a tumor if inoculated during certain environmental conditions. The fact that antigen of B-38 produced a precipitin line next to the antigen well when challenged against its homologous antiserum—but not against antisera of other *A. tumefaciens* strains—suggests that the correlation between virulence and the formation of the precipitin line next to the antigen well may still hold, but that there may be some variation in the nature of the *b* antigen in virulent strains.

These findings are similar to those of Schnatnorst et al. (1964) who reported that virulent isolates differed in antigenic structure. They also found that the line next to the antigen well was associated with virulent isolates (J. E. De Vay, personal communication).

## PHYSIOLOGICAL TESTS

Physiological tests have not appeared satisfactory for differentiating *A. tumefaciens* from *A. radiobacter*. However, several tests have been cited as useful for identification purposes with the agrobacteria.

The physiology of eight California isolates of *A. tumefaciens* and seven of *A. radiobacter* were compared, using a small number of tests to determine whether there were any gross physiological differences between the two species and to determine variation within the species. The physiological tests used in

the study included nitrate reduction (Society of American Bacteriologists, 1957), oxidase test (Stanier et al., 1966), 3-ketoglycoside formation (Bernaerts and De Ley, 1963), and starch hydrolysis (Society of American Bacteriologists, 1957). The production of acid from 17 carbohydrates was tested, using the basal medium described by Hugh and Leifson (1953).

Table 4 shows that virulent and avirulent isolates could not be distinguished on the basis of these tests. The tests also demonstrate the hazard of

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TABLE 4  
ACID PRODUCTION ON VARIOUS CARBON SOURCES AND MISCELLANEOUS REACTIONS BY ISOLATES OF  
*AGROBACTERIUM TUMEFACIENS* AND *A. RADII*

Strain	Arabinose	Arbutin	Gelatin	Glucose	Glycerol	Inulin	Lactose	Lactulose	Meliose	Mannitol	Mannose	Raffinose	Sucrose	Xylose	Miscellaneous reduction	3-Keto- glycolate
<i>A. tumefaciens</i> Ach-1	++	++		++	++	+	+	+	++	++	+	+	++	++	-	+
AI-1	++	++	++	++	++	+	+	+	++	++	+	+	++	++	+	+
II-38	++	++	++	++	++	+	+	+	++	++	+	+	++	++	+	+
II-48	++	++	++	++	++	+	+	+	++	++	+	+	++	++	+	+
CG-1	++	++	++	++	++	+	+	+	++	++	+	+	++	++	+	+
Dah-1	++	++	++	++	++	+	+	+	++	++	+	+	++	++	+	+
PI-1	++	++	++	++	++	+	+	+	++	++	+	+	++	++	+	+
R-12	++	++	++	++	++	-	++	++	+	++	++	+	++	++	+	-
<i>A. radiobacter</i> Rad-0408	++	++	++	++	++	+	++	++	++	++	+	+	++	++	+	+
Rad-0407	++	+	++	++	++	+	+	++	++	++	++	+	++	++	+	+
Rad-B	++	++	++	++	++	+	+	++	++	++	++	+	++	++	+	+
Rad-C	++	++	++	++	++	+	++	++	++	++	++	+	++	++	+	+
Rad-D	++	+	++	++	++	++	+	++	++	++	++	++	+	++	+	+
TT-1	++	++	++	++	++	+	+	++	++	++	+	+	++	++	-	+
TT-2	++	++	++	++	++	+	+	++	++	++	+	+	++	++	-	+

\*The reactions are recorded as ++ trace, + weak, and +++ strong.

taching significant value to any particular test, as there was considerable variation among the isolates. The ability to reduce nitrate varied among strains of a species, and slight differences were noted between species. Most strains produced a positive reaction with all carbohydrates tested except glycogen. They all produced a positive oxidase test and a negative starch hydrolysis test. Isolate R-12 was the only strain which did not produce 3-ketoglycoside.

As pathogenicity has been the only

basis for placing *A. tumefaciens* and *A. radiobacter* in separate species, these data and those of De Ley (1968) and Graham and Parker (1964) suggest there are no taxonomic data available to merit such separation. Even though there may be some serological differences, they alone cannot be used as a basis for speciation. It is unfortunate that the splitting of *A. tumefaciens* and *A. radiobacter*, as with many bacterial groups, has not been based on comprehensive studies.

### DISEASE OCCURRENCE

Disease inception in stone fruit and other trees can occur at any time during the life of the tree as a result of a wound caused by growth, frost cracks, or mechanical injuries. The greatest incidence of disease, however, occurs in the nursery principally during (1) the germination process (as in peaches and almonds) when there is abrasion of radical or epicotyl against the rough seed coat, (2) the harvesting of liners and the subsequent planting in nursery soils, (3) final harvesting of trees for the market when a blade or other device is used to uproot the tree from the soil, and (4) the storing of trees in sawdust-sand healing-in beds. Infection is most serious when it occurs during the harvest period. Although inspectors and nurserymen cooperate to prevent shipping of infected trees, they can eliminate only those having visible symptoms because of their inability to discern diseased trees having incipient infections or which have been contaminated during harvesting. It is not uncommon for a nurseryman to discard 5 to 80 per cent of his trees because of the disease and then to sell the remainder of the so-called healthy trees. It can be safely assumed that if 5 to 80 per cent of the trees were infected and discarded, the apparently healthy, gall-free trees are contaminated, and many will be infected during the har-

vesting period. If infection does not occur during this process, there is an excellent likelihood that it will occur when trees are placed in healing-in beds. An analysis of the materials used in healing-in beds has shown that an incidence of the organism as high as 500 cells per gram of soil is not uncommon. Compostants have greatly helped to reduce the amount of infection occurring in healing-in beds, although not eliminating the disease entirely. Some infection will still occur, since the compost becomes contaminated with the pathogen present in incipient galls and in soil clinging to roots of trees placed in the beds. Rigorous procedures used by nurserymen to eliminate the disease, combined with periodic inspection, have greatly reduced the number of diseased plants set in orchards, but until an effective dip treatment is developed there is no practical method of insuring that trees sold will be disease-free. Infection frequently occurs when trees are injured in the orchard during planting. We have often detected the organism in field soil (also, contaminated soil from the nursery usually clings to the roots). Trees often become infected in the field as a result of injuries caused by cultivation, although this may not adversely affect them (depending upon their age). Crown gall tumors occurring on mature trees generally cause minor

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damage to the trees, because the restrictive effect of the tumor on translocation of solutes is small. When young trees are infected, however, the tumors continue to progress as the trees mature, and by maturity the tumor radiates from the center of the tree to the exterior, and in many cases restricts flow of nutrients in the tree. This causes a serious disruption of translocation, especially when the tumor becomes mori-

bund and, as often occurs, is invaded by heartrot fungi.

Because infection can occur during various stages of the life of the tree, it is difficult to state with certainty whether infection of a tree has occurred in the nursery or in the orchard. This has been a topic of controversy among growers and nurserymen, especially when a large amount of crown gall has been detected in young orchards.

### CHEMOTHERAPEUTIC CONTROL OF CROWN GALL TUMORS

An effective chemotherapeutic treatment using Bacticin® has been developed that successfully eradicates crown gall tumors from many plants including peach, cherry, almond, pear (Schroth and Hildebrand, 1968), walnut, and rose. Bacticin selectively penetrates and eradicates neoplastic tissues. The critical time to use this material to control the disease is during the first several years of tree growth—therefore examination of the crown region of a plant for tumors should include the area below the soil line, as this is the primary site of infection. Many trees in an orchard may be affected by the disease, although growers may not be aware of it until the vigor of the trees has declined. Examination of the crown should be conducted after the first year of planting, using a method which does not cause injuries; currently, the recommended soil removal procedure is the use of an hydraulic system (Ross et al., 1970).

Exposed crown gall tumors are treated by a liberal application of Bacticin to the entire tumor area, with some overlap onto the surrounding healthy tissues. The tumors should be relatively free of soil. To conserve material, some growers remove most of each tumor with a hatchet, although some reports indicate that treatment is less effective when much gall tissue is

removed. The cracks and folds on the rough exterior of a tumor probably help to retain the chemicals, preventing them from flowing into the soil and allowing more to be absorbed by tumor tissue. The tumor should remain exposed at least for several weeks after treatment, since coverage with soil appears to reduce efficacy. Crown gall tumors should be dry at the time of application for better penetration of the oil carrier into the tissues. Although penetration of a tumor 4 inches in diameter occurs within 24 to 48 hours, death may not occur until after 2 or 3 months, or sometimes 4 months in winter.

One application of Bacticin is sufficient to eradicate most tumors. However, tumors over 4½ inches in diameter may sometimes require two applications, especially with pears and cherries where the tumors appear to be more resistant to the toxic action of the hydrocarbons in the material. Extensive testing by growers and farm advisers has shown Bacticin to be highly effective against crown gall on almond, apricot, peach, plum, pear, and cherry. It works equally well against olive knot tumors caused by *Pseudomonas savastanoi*. In 1966, 299 crown gall tumors were treated on stone fruit and pear trees. Of these, 274 were eradicated and 25 had 50 per cent or more of the tumor



tissues killed; most of the 25 that contained live tumor tissue after treatment had been incompletely covered during application (Schroth and Hildebrand, 1968).

When tree crowns are encompassed by tumors, only 33 per cent of the crown area should be covered with Bacticin at one application because complete coverage at one time may damage the tree. However, some browning may occur on healthy tissues adjacent to the tumor. Because translocation of materials occurs through tumorous tissues, their eradication may affect the supply of materials to the adjacent tissues. Also, the tissues immediately surrounding the tumor appear to be more susceptible to the toxic action of the compound, presumably because the disease has some effect on surrounding healthy tissue (Schroth and Hildebrand, 1968). Because high temperatures increase phytotoxicity, it may be necessary to dilute Bacticin by 20 per cent or more under certain climatic conditions.

A long-term effect of using Bacticin to control tumors has been favorable. After 4 years of observation we conclude that Bacticin does not retard callus formation around the tumor site, and that there seldom is evidence of regrowth of the tumor or production of new tumors around the original site.

Crown gall tumors have been treated over the years with other chemicals such as antibiotics (Ark and Sibray, 1957; Dye et al., 1950; Dye, 1952; Sorauer, 1956) and phenolic compounds (Démétriades, 1953; Phillips, 1895; Sorauer, 1956; Stapp, 1961). The phenolics are highly toxic to both plants and animals and their use has not been cleared by the Food and Drug Administration of the U. S. Department of Health, Education, and Welfare. Of the phenolic materials, sodium dinitro-ortho-cresolate (sold commercially as Elgetol), when mixed with methanol, has been used in treatment of crown galls in California (Ark and Scott, 1951).

## FUMIGATION AND SEED TREATMENT

Previous results indicate that *A. tumefaciens* persists for long periods in many soils. Therefore, it would be highly beneficial to reduce or eliminate soil infestation with many crops, such as nursery trees and field-grown roses. Accordingly, several soil fumigants and methods of application were evaluated for the control of crown gall. Because of the possibility that the crown gall bacterium may be introduced into non-infested soil from contaminated plant parts, the effectiveness of treating planting material with disinfectants was also evaluated.

### Field-grown Roses

Cuttings used for starting field-grown roses present an ideal infection court for the crown gall bacterium and therefore roses grown in infested soil often have a high level of crown gall

infection, with attendant financial loss.

In the first tests, chloropierin was applied at rates of 1, 2, and 3 ml per linear foot in a single strip down the center of the bed at a depth of 3½ to 4 inches. The bed was immediately covered with black paper through which the cuttings were inserted. Effect of surface treating cuttings with sodium hypochlorite was also evaluated. Treated cuttings were immersed in a 0.5 per cent solution of sodium hypochlorite for 1 minute just before planting. After about 9 months growth the plants were dug and the percentage of plants with gall was ascertained.

The fumigation reduced crown gall infection but did not effectively control the disease (table 5). However, tumors on plants growing in non-treated soil were much larger than those in treated soil. This may reflect a delayed buildup

or fewer  
#5 or  
more callus  
before infection

TABLE 5  
EFFECT OF IN-THE-FURROW  
FUMIGATION WITH CHLOROPICRIN  
AND SURFACE TREATMENT OF  
CUTTINGS WITH SODIUM  
HYPOCHLORITE ON CROWN GALL  
IN FIELD-GROWN ROSES

Treatment*	Per cent infected plants†	
	Plot 1	
Chloropicrin		
1 ml per foot.....	80.4	
2 ml per foot.....	86.1	
3 ml per foot.....	88.5	
Control.....	88.2	
	Plot 2	
	Cuttings treated	Cuttings not treated
Chloropicrin		
3 ml per foot.....	78.5†	70.6
Control.....	97.3	93.1

\* LSD 5% level = 23.4

† Each value is the mean of 4 replications (approximately 50 plants per replication).

‡ Each value is the mean of 4 replications (approximately 100 plants per replication).

in the bacterial population and later infection. Surface treatment of the cuttings had no effect on disease severity (table 5).

Because of the slight reduction in disease severity obtained with an in-the-furrow fumigation, a second trial was conducted the following year. In this test, solid blocks of plants were treated and then tarped with polyethylene. The fumigant was a mixture of  $\frac{3}{8}$  chloropicrin and  $\frac{1}{8}$  methyl bromide applied at a rate of 320 pounds per acre at a depth of 6 inches.

This solid block tarped fumigation resulted in an appreciably greater re-

duction in disease incidence than did the in-the-furrow application, but still did not result in the desired degree of control (table 6).

These two plots provide an excellent example of seasonal variations of the crown gall disease. The second trial was on the same land as the first; yet disease severity in the controls varied from 88 to 93 per cent of trees infected the first year to 86 per cent the second. This could have been caused by many factors: soil moisture and temperature during rooting, condition of the cuttings, or seasonal changes in the population of the organism.

### Almond Seedlings

In this trial, soil fumigation and seed treatment were evaluated, using a split-plot design. The fumigant used was a mixture of chloropicrin ( $\frac{3}{8}$ ) and methyl bromide ( $\frac{1}{8}$ ) at rates of 396 and 792 pounds per acre, followed by tarping with polyethylene. The seed treatments were mercuric chloride at 1 ppm plus a wetting agent (3 ounces household detergent per 10 gallons of water for 15 minutes, and 1 per cent sodium hypochlorite plus wetting agent for 15 minutes). Almond seed, previously stratified, was treated on February 25 and planted on March 5 after 4 days of pre-soaking. Trees were pulled and the percentage of crown gall determined on December 17.

~~Treatment of seed had no influence on the incidence of disease~~ (table 7). Soil fumigation significantly reduced crown gall, but as with the roses, it did

Pre-soaking  
would have  
diluted out  
chem.

TABLE 6  
EFFECT OF SOLID-BLOCK FUMIGATION WITH TARPING ON CROWN GALL  
OF FIELD-GROWN ROSES

Treatment	Number of plants observed	Number galled	Per cent gall	Per cent infection
Fumigation-320 lb. per acre*	396	26	11	66
Control.....	397	75	26	93

\* Data based on 6 replications. Material was  $\frac{3}{8}$  chloropicrin and  $\frac{1}{8}$  methyl bromide.

not provide a satisfactory level of control (table 8). The failure to satisfactorily control the disease did not appear to be caused by an inadequate rate of fumigant application. Doubling the rate from 396 to 792 pounds per acre did not have any effect on crown gall severity, indicating that this was not the limiting factor.

TABLE 7  
EFFECT OF SEED TREATMENT  
ON CROWN GALL OF ALMOND  
SEEDLINGS

Treatment	Infected plants
	Per cent
Mercuric chloride—0.1% for 15 minutes	14.7*
Sodium hypochlorite—1% for 15 minutes	15.5
Control	17.5

\* Each figure is the mean of 12 replications, at 50 to 250 trees per replication. Difference not statistically significant.

These tests show that soil fumigation can reduce the incidence of crown gall, particularly when solid application and tarping is employed. Even under these conditions, however, the disease was not effectively controlled. It is interesting to note that with two very different hosts, and in two different areas, the percentage of reduction was similar—this suggests that these data provide a good picture of the effect of soil fumigation on crown gall.

## SUMMARY AND CONCLUSIONS

*Agrobacterium tumefaciens* was isolated from various California soils, using either a selective medium or a carrot discs trap technique. The bacterium appears to survive for long periods in these soils and the population ratio of *A. tumefaciens* to *A. radiobacter* varied from 1:13 to 1:500. Many strains of *A. tumefaciens* aggregated around roots of various seedling plants when the seedlings were placed in water containing bacterial cells. There ap-

TABLE 8  
EFFECT OF SOIL FUMIGATION  
ON CROWN GALL OF ALMOND  
SEEDLINGS

Treatment	Infected plants	Reduction
	Per cent	Per cent
Chloropicrin-methyl bromide		
396 lb per acre	11.2*	49
792 lb per acre	12.5†	43
Control	21.8	

\* Each figure is the mean of four replications (300 to 600 trees per replication).

† These values differ significantly from the control at the 5% level.

The reasons for this lack of control are not known. The fumigant has been shown in plastic bag tests to be highly effective against the bacterium (Munnecke and Ferguson, 1960). It also has been effective in controlling *A. rhizogenes*, a related bacterium (Munnecke et al., 1963). However, *A. rhizogenes* is a nutritionally fastidious organism requiring vitamins, and it may be a poor soil invader and competitor. Our results (table 8) suggest that in the field a low percentage of the *A. tumefaciens* cells survive the treatment, and that the bacterium increases rapidly in fumigated soil to a level which can cause appreciable disease if a susceptible host is planted. The data of Deep et al. (1968) and Dickey (1962) support this supposition.

peared to be specific sites for aggregation: the root tap zone repulsed bacteria, but root hairs served as attractant.

Serological studies using the Ouchterlony gel-diffusion test suggested that virulent isolates possessed (in contrast to *A. radiobacter*) a distinct antigen that formed a precipitin line next to the antigen well. This antigen appeared to be closely associated with the cell wall, and was separable from other

antigens by filtration with a 0.22- $\mu$  Millipore filter. Physiological tests proved of no value in differentiating virulent and avirulent agrobacteria.

The occurrence of disease in stone fruits and the application of cultural practices to avoid the disease and a chemotherapeutant for eradication of crown gall tumors are discussed. Fumi-

gation of soil with chloropierin and methyl bromide reduced disease incidence but did not economically control the disease. Treated nursery soil was subsequently found to harbor *A. tumefaciens* as a result of incomplete eradication, or contamination by wind-blown dust, tools, or irrigation water containing the organism.

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# Study Dec. E-4

## AN ABSTRACT OF THE THESIS OF

Arthur Robert Anderson for the degree of Doctor of Philosophy  
in Plant Pathology and Soil Microbiology presented on July 29, 1977

Title: TAXONOMY AND HOST SPECIFICITY OF THE GENUS  
AGROBACTERIUM

Abstract approved: \_\_\_\_\_

\_\_\_\_\_  
Larry W. Moore

\_\_\_\_\_  
Raymond Seidler

One hundred seventy-eight Agrobacterium isolates, 120 United States (U.S.), 26 Australian and 32 of miscellaneous origin, were characterized for their reactions to 20 biochemical and physiological tests. Isolate pathogenicity and degree of host specificity, utilization of nopaline and octopine, and sensitivity to agrocin-84 also were determined. The U.S. isolates were quite similar, but not identical in character to the 26 Australian isolates. Nearly all the isolates were separated by 15 diagnostic tests into two homogeneous groups, designated 3-ketolactose positive [K(+)] and 3-ketolactose negative [K(-)]. Sixteen isolates did not conform to either group description. Isolates of the species A. tumefaciens and A. rubi were biochemically, physiologically and pathologically indistinguishable. Five of eight confirmed A. rhizogenes isolates induced tumors in addition to hairy root and were all K(-) in character. A taxonomic proposal

for the genus Agrobacterium is presented wherein two species would be recognized.

All agrobacteria were inoculated to 11 known crown-gall hosts to determine their host range. The host specificity of all isolates varied greatly with no isolate infecting all host plants. Sixty-six percent of the pathogenic isolates infected six to eight of 11 hosts tested while 3% infected only the host plant from which they were originally isolated (homologous host). However, the host range of an isolate was not influenced by the plant of isolation. Evidence for change in an isolate's host specificity pattern while in culture is presented and discussed relative to its relationship with the Ti plasmid. Tomato and datura were infected by more of the pathogenic agrobacteria (81% each) than any other of the 11 host species tested, thus serving as the best indicators of an isolate's pathogenicity.

Low correlations were observed between all combinations of five isolate characters; pathogenicity, 3-ketolactose reaction, nopaline and/or octopine utilization and agrocin-84 sensitivity. Of 32 possible permutation groups of the five characters examined, isolates belonging to 25 groups were identified. Isolates were randomly distributed among all 25 groups regardless of geographic origin or plant species of isolation. Possible reasons for the lack of high correlations between the plasmid-coded characters are discussed.

### Biochemical and Physiological Tests

Twenty selected tests were compared for their reported differentiating capabilities (cf. Table 4). Test media were incubated at 28 C unless stated otherwise. Liquid test media were inoculated with 0.1 ml of a cell suspension containing approximately  $10^6$  colony forming units/ml. Slants were inoculated directly by streaking from the working cultures. Plates were replica plated (26) from a 24-36 hour old master plate. All isolate reactions were recorded after 14 days incubation, except liquid tube and slant media tests which were incubated for an additional week and then recorded. The individual test procedures are listed below.

- 1) 3-Ketolactose production (4).
- 2) Oxidase (13). Development of a purple coloration within 10 seconds was considered positive.
- 3) Litmus milk (Difco) reaction. Potentiometric measurement of the pH reaction also was measured.
- 4) Sodium chloride tolerance. Nutrient agar (Beef extract, 3.0 g/l; peptone, 5.0 g/l; and agar, 1.8%) supplemented with sodium chloride at a concentration of 1, 2, 3, 4, or 5% (w/v) was used.
- 5) Utilization of Malonate (17). A blue substrate coloration was positive.

- 6) Utilization of Citrate (25). A blue substrate coloration was positive.
- 7) Sodium selenite medium (10). The sodium selenite was added before autoclaving. Red coloration of the colony was positive.
- 8) Acid production from erythritol (9).
- 9) Growth on L-tyrosine (16).
- 10) Growth on sodium-D, L-lactate. The mineral basal medium of Kersters et al. (16) was used. A 1% (w/v) filter-sterilized solution of sodium-D, L-lactate and bromocresol purple (0.0016%, w/v) were added after autoclaving. Growth was measured as increased turbidity.
- 11) Growth on Schroth et al. medium (24).
- 12) Growth on New and Kerr medium (20).
- 13) Growth on DIM medium (12).
- 14) Growth on ferric ammonium citrate broth (10). Pellicle formation was positive.
- 15) Growth at pH 11.6. A mannitol (10.0 g/l)-yeast extract (5.0 g/l) medium (1.8% agar) was autoclaved and the pH adjusted to 11.6 with 40% (w/v) NaOH.
- 16) Maximum growth temperatures. Nutrient agar plates (see #4) were inoculated and incubated in thermostatically controlled, warm-air incubators at 25, 30, 34 and 40 C for up to two weeks.

- 17) Growth on glycerophosphate medium. Medium I of Riker et al. (22) was used. A white precipitate was positive.
- 18) Growth on raffinose medium (7).
- 19) Absorption of aniline blue (10).
- 20) Oxidation-reduction potential (8).

### Pathogenicity of Isolates

The ability of all isolates to induce tumors on 12 host species was tested using previously described methods (3). Hairy root induction by A. rhizogenes was determined by the carrot assay (18).

## RESULTS

### Biochemical and Physiological Tests

Sixteen of the 20 biochemical and physiological tests used separated 72 United States (U.S.) and 26 Australian Agrobacterium isolates into two groups designated 3-ketolactose positive [K(+)] and 3-ketolactose negative [K(-)], similar to those described previously (13, 16, 21, 24). An additional 80 Agrobacterium isolates were tested further and similarly separated by these 16 diagnostic tests (Table 3). The remaining four tests (raffinose, aniline blue, DIM, and oxidation-reduction potential) gave extremely variable results and

Table 3. Characterization of 136 *Agrobacterium* isolates for reaction to 16 biochemical tests. Results are given as percentage of isolates with the 3-ketolactose positive [K(+)] strain or negative [K(-)] strain reaction.

Biological and physiological tests	K(+) <sup>a</sup>	% <sup>b</sup>	K(-) <sup>c</sup>	% <sup>d</sup>
3-Ketolactose	+	100	-	100
Ferric ammonium citrate	+	100	-	100
NaCl tolerance	3-4%	98.6	1%	100
L-tyrosine	-	100	+	93.8
Schroth et al.	+	97.1	-	98.5
Erythritol	-	98.5	+	95.3
New and Kerr	-	98.5	+	92.4
Oxidase	+	98.6	-	92.2
Citrate	-	97.0	+	92.3
Na-D, L-lactate	+	100	-	84.8
Malonate	-	100	+	83.1
pH 11.6	+	97.1	-	86.7
Litmus milk	b	100	a	76.6
Na selenite	+	100	-	74.2
Glycerophosphate	+	100	-	70.6
Maximum growth temperature	40 C	84.8	34 C	90.3

<sup>a</sup> K(+) strain reactions

<sup>b</sup> Percentage of isolates positive for the 3-ketolactose test which have the test reactions for a given test corresponding to the K(+) strain reactions.

<sup>c</sup> K(-) strain reactions

<sup>d</sup> Percentage of isolates negative for the 3-ketolactose test which have the test reactions for a given test corresponding to the K(-) strain reactions.

Table 4. Comparison of diagnostic tests results for the 3-ketolactose positive and negative *Agrobacterium* strains as reported in several recent taxonomic references.

Diagnostic tests	Literature references with reported biochemical and physiological test results			
	Strain reactions <sup>a</sup>		physiological test results	
	(US isolates)	Keane et al. (13)	Kerstens et al. (16)	White (30)
	K(+) <sup>b</sup> K(-) <sup>b</sup>			
3-Ketolactose	+	Same results	Same results	Same results
Oxidase	+	Same results	Variable	All positive
Litmus milk	b	Same results	Same results	Same results
NaCl tolerance	3-4% 1%	Not tested	Not tested	Same results
Citrate	-	Same results	Same results	All positive
Erythritol	-	Same results	Not tested	Same results
Na selenite	+	Same results	Variable	Not tested
L-tyrosine	-	Not tested	Same results	Not tested
Na-D, L-lactate	+	Not tested	Same results <sup>c</sup>	Not tested
Schroth et al.	+	Not tested	Not tested	Not tested
New and Kerr	-	Not tested	Not tested	Not tested
Malonate	-	Same results	Not tested	Same results
Ferric ammonium citrate	+	Same results	Same results	Not tested
pH 11.6	+	Not tested	Not tested	Not tested
Glycerophosphate	+	Same results	Not tested <sup>d</sup>	Not tested
Maximum growth temp.	40 C 34 C	Not tested	Not tested	Similar results <sup>c</sup>
Raffinose	V	Not tested	Not tested	K(+), +; K(-), -
Araline blue	V	Same results	Same results	Not tested
Oxidation-reduction	V	Same results	Not tested	Not tested
DIM	V	Not tested	Not tested	Not tested

<sup>a</sup> Abbreviations for the test reactions are: + = positive; - = negative; b = alkaline; a = acid; and V = variable.

<sup>b</sup> K(+) = 3-ketolactose positive strain and K(-) = 3-ketolactose negative strain.

<sup>c</sup> Only the D isomer was tested.

<sup>d</sup> A white precipitate was not tested for on glycerophosphate medium.

<sup>e</sup> The relative temperature studies were the same, but the absolute temperatures were not.

Table 5. Biochemical and physiological reactions<sup>a</sup> of 16 intermediate *Agrobacterium* isolates to 14 tests.

Isolate	Source	Pathogenicity <sup>b</sup>	3-Ketolactose	Oxidase	Lidius milk	NaCl tolerance	Citrate	Erythritol	Na selenite	L-tyrosine	Na-D, L-lactate	Schroth et al.	New and Kerr	Malonate	Ferric ammonium citrate	pH 11.6
H7/72	<u>Asalea</u>	-	-	-	a	5	-	+	+	+	+	-	+	-	+	+
E3/73	<u>Dahlia</u>	-	-	+	b	4	+	+	+	+	+	+	+	-	+	-
L47/73	Cherry, Massard	-	-	+	b	1	+	-	+	+	+	-	-	+	-	-
L180/73	Soil	-	-	-	a	5	-	+	+	+	+	-	-	-	+	+
L241/73	Soil	-	-	+	b	1	+	+	+	-	+	-	-	-	-	-
W1/73	<u>Enonymus</u>	+	-	+	b	1	+	-	+	-	-	-	-	+	-	+
W2/73	<u>Enonymus</u>	+	-	+	b	1	+	-	+	-	-	-	-	+	-	-
P6/73	Baby's breath	-	-	+	b	5	-	+	NT	-	+	+	-	-	-	NT
P8/73	Baby's breath	-	-	+	b	1	+	+	NT	+	+	+	+	-	+	NT
B10/75	Apple, Malling	-	-	+	b	1	-	+	NT	+	-	+	+	-	+	NT
B12/75	Apple, Malling	-	-	+	b	1	-	+	NT	+	-	+	+	-	+	NT
AT181a		+	-	+	b	4	-	-	NT	-	+	+	-	-	+	NT
G362		+	-	+	b	5	-	+	NT	-	+	+	-	-	+	NT
11325 I		+	-	+	b	1	+	-	NT	-	-	-	-	+	-	NT
11325 II		+	-	+	b	1	+	-	NT	-	-	-	-	+	-	NT
13335		+	-	+	b	3	+	-	NT	-	+	-	-	+	-	NT

Characteristic reactions for the two strains of *Agrobacterium*

K(+) <sup>c</sup>	+	+	+	+	b	3-4%	-	-	+	+	+	+	+	+	+	-
K(-) <sup>c</sup>	-	-	-	-	a	1%	+	+	-	+	-	-	-	-	-	-

<sup>a</sup> Abbreviations for the test reactions are: + = positive; - = negative; b = alkaline; a = acid; and NT = not tested.

<sup>b</sup> Pathogenicity was tested on Bonny Best tomatoes.

<sup>c</sup> K(+) and K(-) denote the two strains of *agrobacteria*, one 3-ketolactose positive and the other 3-ketolactose negative. The characteristic test reactions of each strain are given.

Study Dec. E-5

# Laboratory Guide for Identification of Plant Pathogenic Bacteria

## 2nd Edition

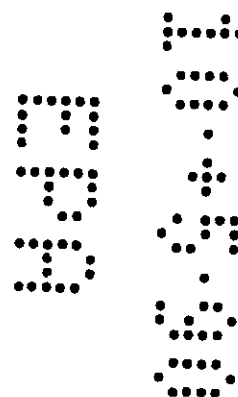
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St. Paul, Minnesota



## II. Gram-Negative Bacteria

### A. Agrobacterium

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#### 1. INTRODUCTION

The genus Agrobacterium belongs to the family Rhizobiaceae (19). Members of this family are aerobic, Gram-negative. The cells are normally rod-shaped (0.6-1.0  $\mu$ m by 1.5-3.0  $\mu$ m), occur singly or in pairs, without endospore, and motile by one to six peritrichous flagella. Considerable extracellular polysaccharide slime is usually produced during growth on carbohydrate-containing media. Upon infection of plants, some strains of Agrobacterium induce abnormal cell proliferations (hyperplasias) which result in tumor formation in the case of the crown gall disease, and excessive adventitious roots, as in the hairy root disease; however, many strains are nonpathogenic. Agrobacteria are differentiated from the symbiotic Rhizobiaceae by their inability to induce nodule formation and to fix atmospheric nitrogen. According to Skinner (48), agrobacteria can be differentiated from rhizobia by the Nile blue test because Agrobacterium reduces the dye more strongly to its colorless state.

The species nomenclature of Agrobacterium, as presented in the Approved Lists of Bacterial Names (47) and the last edition of Bergey's manual (29), is still based on phytopathogenic behavior. Those strains causing crown gall are placed in Agrobacterium tumefaciens, those causing hairy root in A. rhizogenes, those causing cane gall on Rubus spp. in A. rubi and nonpathogens in A. radiobacter. A major problem with this nomenclature is the fact that genes for pathogenicity are carried on large Ti (tumor-inducing) or Ri (root-inducing) plasmids (34,39,53,58) which can be lost or transferred (24,55) to a nonpathogenic strain, which in turn becomes pathogenic. Thus, the species status of an individual strain can be changed according to the presence or absence of these plasmids. Furthermore, no correlation exists between the current nomenclature and the taxonomic structure based on morphological, physiological and genotypical traits of the genus (29). Results obtained from analysis of phenotypic characters (18,22,27,30,50,57), chromosomal DNA (11,12) and comparison of electrophoretic protein patterns (28) show that the genus Agrobacterium consists of at least three taxonomic groups (2,22,25,27,33). The two largest groups correspond to biovar 1 and biovar 2 and were proposed to be raised to the species level (18). Although this proposed nomenclature appeared to be a good solution to objectively... differentiate the agrobacteria, this speciation scheme is not officially accepted at present (29). Consequently, to avoid confusion, we shall follow the classical nomenclature presented in Bergey's manual (29).

#### 2. ISOLATION TECHNIQUES USING SELECTIVE MEDIA

##### a. Plant Material

Agrobacterium strains can be isolated from tumors, vascular fluids (e.g. grape), above and below-ground surfaces of symptomless plants, soil and water. In contrast to many bacterial diseases of plants, infected crown gall tissue does not contain masses of bacteria, and a bacterial ooze does not develop.

Because *A. tumefaciens* populations in dark, decomposing tumor tissue are low, young, actively growing galls which contain white tissues should be used for isolation. The gall surface should be washed and the brown, dead tissues discarded to eliminate many secondary bacteria. Cut two to three small subsamples from different locations within the tumor to increase the probability of a successful isolation. Dice the tumor tissue into about 2 by 2 mm pieces and place in 1-2 ml of distilled water for 30 minutes or longer to allow the bacteria to move into the liquid. Both pathogenic and non-pathogenic agrobacteria are typically isolated from tumors (Table 1).

Surface sterilization of the gall tissue for 10-20 minutes in 1% sodium hypochlorite solution (or 20% household bleach) prior to isolating is sometimes done. However, the disinfectant may penetrate into the tissue and kill the pathogen; to reduce this risk, rinse the treated tissues with several changes of sterile water before dicing the tissue. Thorough washing of the gall exterior, removal of dead gall tissue, and use of selective media reduces the need for surface sterilization. General comments about the selectivity and results obtained from studies using different selective media are presented in Table 2.

The selective media we currently use are: Brisbane and Kerr medium 1A (7) which selects for biovar 1 strains (Plate 1, Fig. 2), medium 2E (7) which favors growth of biovar 2 strains (Plate 1, Fig. 3), and a medium developed by Roy and Sasser (44) which selects for biovar 3 strains (Plate 1, Fig. 4). Because more than one strain of *Agrobacterium* can cohabit the same tumor, it may be necessary to use all three media when attempting to determine the predominant biovar. The distribution of biovars varies from one geographic region to another (Table 1).

#### b. Soil

Use of selective media is especially helpful for the isolation of *Agrobacterium* strains from soil because these strains compete poorly with other soil microbes when plated on nonselective media. Even so, some colonies on the isolation plates will not be agrobacteria and the sensitivity of the selective media is limited to about  $10^3$  colony forming units (CFU)/g soil. Higher concentrations of soil dilutions contain more debris and other microorganisms which decrease the selectivity of the media. The isolation procedure commonly used consists of spreading 0.1 ml of 10-fold serial dilutions of soil or extracts of tumor tissue over a solid medium with an L-shaped glass rod. Inoculated plates are subsequently incubated at 27 C.

Single colonies from a selective medium can harbor strains of different biovar affiliations as well as other bacteria, and must always be cloned for purity. To clone for purity, individual colonies from selective media are suspended in sterile distilled water and streaked onto PDA. Single colonies from PDA are again selected and streaked. Repeat the process until all the colonies appear homogeneous. On PDA, *Agrobacterium* strains grow well, and their colonies are generally easily distinguished from other species because they are convex, glistening, circular with an entire edge, and white to beige in color.

Table 1. Geographic distribution of predominant biovars of *Agrobacterium*

Location	Biovar	Number of strains		Selecti
		Pathogens	Nonpathogens	
Australia (23)	1	0	112	D, E
	2	85	15	D, E
Canada (13)	1	3	---	D
	2	27	---	D
Greece (42)	1	13	6	Nutri
	2	23	2	Nutri
	Intermediate	4	3	Nutri
Hungary (50)	1	6	---	
	2	38	---	
	3	25	---	
Netherlands (H. Miller, personal communication)	1	90	Rare	A, C
	2	0	Rare	A, C
New Zealand (49)	1	123	64	A, B
	2	1	39	A, B
	1-2	0	36	A, E
United States				
Georgia (6)	1	16	26	C, E
	2	7	36	C, E
Maryland (1)	1	0	9	A, E
	2	2	13	A, E
Oregon (2-36)	1	92	167	C, E
	2	460	736	C, E
	Intermediate	7	9	C, E
Pennsylvania (L. Forer, personal communication)	1	6	...	A, E
	2	60	...	A, E
	Intermediate	...	...	A, E
South Carolina (1)	1	...	38	A, E
	2	43	102	A, E
Tennessee (1)	1	37	25	A, E
	2	123	15	A, E

A = Clark (8); B = D1 (20); C = New - Kerr (41); D = Patel (43); E = et al. (45), and F = D1M (38).

Table 2 - Selectivity of the media for isolation of the major biovars of Agrobacterium.

Medium	Major biovar recovered	Comments
Patel (43)	1 and 2	Discriminated poorly against the normal soil microflora. However, all agrobacteria tested by Brisbane and Kerr grew (7).
Schroth et al. (45)	1	Excluded 99% of other species growing on nonselective media; efficiency of recovery was 38%. Reducing antibiotics increased efficiency of recovery; some biovar 2 grew when ammoniacal nitrogen was used; use of this selective medium as a liquid enrichment medium allowed detection of <u>A. radiobacter</u> in 28 soils (45). Omission of streptomycin helps improve recovery of some biovar 1 strains that are sensitive to this antibiotic (7).
Clark (8)	1	Biovar 2 also grew, but poorly; biovar 3 were recovered as well. The manganese sulphate concentration had to be reduced by half to allow growth of <u>Agrobacterium</u> strains in Australia (7).
D1 (20)	1 and 2	Biovars 1, 2, and 3 were recovered on Kado's modified D1 medium (D1M) (38) (Moore, L. W., unpublished).
New - Kerr (41)	2	Nitrate was reported to prevent growth of <u>A. rhizogenes</u> and <u>Rhizobium trifolii</u> . However, Anderson (2) found that 26/27 <u>Rhizobium</u> strains and <u>A. rhizogenes</u> did grow in the presence of nitrate.
1A, 2E and 3DG (7)	1, 2 and 3	Each of these media was reported (7) as highly selective and efficient (99%) for the respective biovars. However, tartaric acid, which is essential for selectivity for the biovar 3 medium, is not utilized by biovar 3 strains from chrysanthemum (4).
Roy - Sasser (44)	3	Very selective for biovar 3 strains and effective when isolating from soil. Colony morphology is very distinct. (Moore, L. W., unpublished).

c. Recipes for selective media.

Medium 1A for biovar 1 (7)

	<u>Per L</u>
L (-) arabinol	3.04 g
NH <sub>4</sub> NO <sub>3</sub>	0.16 g
KH <sub>2</sub> PO <sub>4</sub>	0.54 g
K <sub>2</sub> HPO <sub>4</sub>	1.04 g
Sodium taurocholate	0.29 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.25 g
Agar	15.00 g
Crystal violet, 0.1% (w/v) aqueous	2 ml

Autoclave, cool to about 50 C, then add filter-sterilized cycloheximide (1.0 ml of 2% solution) and Na<sub>2</sub>SeO<sub>3</sub> (6.6 ml of 1% solution).

Medium 2E for biovar 2 (7)

	<u>Per L</u>
NH <sub>4</sub> NO <sub>3</sub>	0.16 g
Erythritol	3.05 g
KH <sub>2</sub> PO <sub>4</sub>	0.54 g
K <sub>2</sub> HPO <sub>4</sub>	1.04 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.25 g
Sodium taurocholate	0.29 g
Yeast extract, 1% (w/v) aqueous	1 ml
Malachite green, 0.1% (w/v) aqueous	5 ml
Agar	15.00 g

Autoclave, cool to 50 C, then add filter-sterilized cycloheximide (1.0 ml of 2% solution) and Na<sub>2</sub>SeO<sub>3</sub> (6.6 ml of 1% aqueous).

Roy-Saxer medium for biovar 3 (44)

	<u>Per L</u>
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2 g
K <sub>2</sub> HPO <sub>4</sub>	0.9 g
KH <sub>2</sub> PO <sub>4</sub>	0.7 g
Adonitol	4.0 g
Yeast extract	0.14 g
NaCl	0.2 g
H <sub>3</sub> BO <sub>3</sub>	1.0 g
Agar	15.0 g
Chlorothalonil (Bravo 500), 4% (w/v) aqueous	0.5 ml

Adjust to pH 7.2, autoclave, cool to 50 C and add aseptically the following (after dissolving separately in a few ml of distilled water and filter sterilizing):

Triphenyltetrazolium Chloride	80 mg
D-cycloserine	20 mg
Trimethoprim (with 1 drop HCl to the distilled water)	20 mg

Colonies of biovar 3 are countable after four days of incubation at 27 C. They will have dark red centers with white edges. Comparison to a known isolate plated at the same time is recommended.

### 3. DIFFERENTIATION OF COMMONLY ISOLATED BIOVARS

The following diagnostic tests (Table 3) separate the strains of Agrobacterium into three biovars. As stated in the introduction, the presently recognized Agrobacterium species are not classified biochemically but rather on their pathogenic characters; therefore, these diagnostic tests will not differentiate between pathogens and non-pathogens.

Table 3. Major differential characteristics of the biovars of the genus Agrobacterium.

Diagnostic test	Biovar		
	1	2	3
3-Ketolactose production	+	-	V
Growth in 2% NaCl	+	-	+
Growth at 35 C	+	V-	V
Action on litmus milk	ALK	AC	ALK
Acid from:			
Sucrose	+	-	V
Erythritol	-	+	-
Melezitose	+	-	-
Alkali from:			
Malonic acid	-	+	+
L-tartaric acid	-	+	+
Propionic acid	V	-	-
Mucic acid	-	+	-
Ferric ammonium citrate	+	-	-
L-tyrosine utilization	-	+	-
Oxidase reaction	+	V-	V
Citrate utilization	V-	+	+

Because genetic mutation and recombination likely occurs with Agrobacterium strains in nature, we should expect to isolate some strains with phenotypic traits that are intermediate between the designated boundaries for the different biovars. However, their frequency of occurrence seems to be low relative to the presently recognized biovars.

### 4. DIAGNOSTIC MEDIA AND TESTS

The following general procedures apply to all of the following recipes:

Use distilled or deionized water to prepare the media. Liquid test media are inoculated with 0.1 ml of starter culture (about 48 h old, washed once in saline and diluted 100-fold to provide  $10^5$  to  $10^6$  CFU per test medium).

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The starter culture is grown for 24-48 h on NGA slants (see 1) p. 3) by streaking from working cultures (working cultures are grown on either PDA or YDC slants, and kept at 4 C). Diagnostic test responses are recorded after 14 days incubation at 27 C, except for tests performed in liquid medium or on slant media which are incubated an additional week before recording.

a. PDA.

Difco dehydrated, prepare as recommended by manufacturer and supplemented with 0.5%  $\text{CaCO}_3$ .

b. 3-katolactose test (5)

Smear bacterial inoculum over about a 1.0 cm-diameter spot on medium containing 1%  $\alpha$ -lactose, 0.1% yeast extract, and 2% agar. Four to six strains can be applied to the same plate. Incubate the plate at 27 C for 2 days. Then, flood the agar surface with a shallow layer of Benedict's reagent and leave at room temperature. If 3-katolactose is present, a yellow ring of  $\text{Cu}_2\text{O}$  becomes visible around the cell mass in about one hour (Plate 1, Fig. 5).

Benedict's reagent: Dissolve 173 g of sodium citrate and 100 g of anhydrous sodium carbonate in 600 ml of distilled water with heating (filter the resulting solution if a precipitate forms). Dissolve 17.3 g of cupric sulfate in 150 ml distilled water. Slowly add the cupric sulfate solution into a large beaker containing the sodium citrate-sodium carbonate solution, while stirring constantly. Dilute to one liter.

c. Growth and pigmentation in ferric ammonium citrate broth (16)

	per L
Ferric ammonium citrate	10.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g
$\text{K}_2\text{HPO}_4$	0.5 g
$\text{CaCl}_2$	0.2 g

Adjust to pH 7.0 before autoclaving. Inoculate the culture tubes containing the broth and incubate in a stationary position. Biovar 1 strains produce a reddish brown pellicle at the surface of the medium (Plate 1, Fig. 6). Auxotrophs may require media supplements of 0.1% L(-) glutamic acid or 0.001% yeast extract to grow.

d. Sodium chloride tolerance (2,22)

Inoculate NGA slants containing 1, 2, 3, 4 and 5% (w/v) sodium chloride and check for growth.

e. Maximum growth temperature (2)

Inoculate NGA slants and incubate for 10 days at temperatures ranging from 28 to 37 C before assessing for growth.

f. Acid production from erythritol, D(+) melezitose and sucrose (15)

Basal medium	per L
$\text{NH}_4\text{H}_2\text{PO}_4$	1.0 g
KCl	0.2 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g

Yeast extract 1.0 g  
 Bromthymol blue, 1% (w/v) in 50% ethanol 3.0 ml  
 Adjust pH to 7.1 with 1N NaOH before adding agar  
 Agar 1.5 g  
 Autoclave for 15 minutes at 121 C.

Add 1 part of filter-sterilized 10% (w/v) erythritol, melezitose or sucrose solution to 9 parts sterile and cooled basal medium; then dispense aseptically about 4 ml of medium to sterile plugged tubes. Development of a yellow color in the medium indicates production of acid from the oxidation of erythritol, melezitose or sucrose.

g. Utilization of L-tyrosine (30)

<u>Basal medium</u>	<u>per L</u>
L-tyrosine	0.40 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.25 g
Na <sub>2</sub> HPO <sub>4</sub>	0.48 g
KH <sub>2</sub> PO <sub>4</sub>	0.55 g
NaCl	0.25 g
CaCl <sub>2</sub> ·2H <sub>2</sub> O	25.00 mg
Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	4.00 mg
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.16 mg
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.08 mg
K <sub>3</sub> BO <sub>3</sub>	0.50 mg
MnSO <sub>4</sub> ·H <sub>2</sub> O	0.40 g
Yeast extract	2.50 mg

Adjust to pH 7.0, autoclave, inoculate culture tubes and observe for growth (turbidity).

h. Oxidase test (31)

For inoculum use 24-hour-old cultures grown on nutrient agar supplemented with 1% glucose. Rub a small loopful of the inoculum on a filter paper impregnated with 1% (w/v) aqueous tetramethyl-p-phenylenediamine dihydrochloride solution\* (freshly made). The strain is rated oxidase-positive if a purple color develops within 10 seconds (Plate 1, Fig. 7), delayed positive if coloration develops within 10-60 seconds; and negative if no color develops after 60 seconds. A platinum loop is recommended since traces of iron can catalyze the oxidation of the phenylenediamine compound.

\* Commercial oxidase strips are available; some investigators prefer dimethyl to tetramethyl.

i. Citrate utilization (46)

Cultures are inoculated to sodium citrate agar slants of the following composition:

	<u>per L</u>
NaCl	5.0 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2 g
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	1.0 g
K <sub>2</sub> HPO <sub>4</sub>	1.0 g

Sodium citrate (anhydrous)	2.0 g
Agar	20.0 g
Bromthymol blue, 1% (w/v) in 50% ethanol	15.0 ml

Dissolve the salts, add indicator and adjust the medium to pH 6.8. Add the agar, and heat the suspension to melt the agar. Dispense the medium in test-tubes, autoclave at 121 C for 15 minutes and slant the tubes to cool. After 24-48 h, the inoculated medium turns to a deep Prussian blue if citrate has been utilized (Plate 1, Fig. 8).

j. Action on litmus milk (2):

Difco dehydrated, prepare as recommended by manufacturer. Acid production turns the milk red whereas alkaline turns the milk blue (Plate 2, Fig. 1)

k. Alkali from malonic acid (27):

	per L
$(\text{NH}_4)_2\text{SO}_4$	2.0 g
NaCl	2.0 g
$\text{K}_2\text{HPO}_4$	0.6 g
$\text{KH}_2\text{PO}_4$	0.4 g
Yeast extract	0.1 g
Malonic acid, sodium salt	3.0 g
Bromthymol blue, 1% (w/v) in 50% ethanol	2.5 ml

Adjust to pH 7.0, dispense 3-4 ml of medium in test tube before autoclaving. Inoculate the medium and incubate at 27 C. The medium will turn blue when alkali is produced (Plate 2, Fig. 2).

l. Alkali from mucic acid, propionic acid or L-tartaric acid (27)

basal medium	per L
$\text{NaNH}_4\text{PO}_4$	0.5 g
$\text{NaH}_2\text{PO}_4$	0.17 g
KCl	0.2 g
Bromthymol blue, 1% (w/v) in 50% ethanol	2.5 ml

Adjust to pH 7.0, dispense 4.5 ml basal medium in test-tube before autoclaving. Then, aseptically add 0.5 ml of filter sterilized 1% solution of either L-tartaric acid, propionic acid or mucic acid previously neutralized with NaOH.

After inoculation, incubate at 27 C for about two weeks. The medium will turn blue when alkali is produced.

## 5. PATHOGENICITY TESTS

Although a great variety of dicotyledonous and some gymnospermous plants can be transformed by agrobacteria, host specificity is the rule for individual strains of Agrobacterium (2,3,34). No host was infected by more than 81% of the pathogenic strains in the study of Anderson and Moore (3) but was the host range of a strain predictably determined by the plant from which the pathogen was isolated (2). Genes on the Ti-plasmid have now been shown as the primary determinant of host range (34,52).

a. Preparation of inoculum

Prepare an inoculum containing  $10^6$  to  $10^7$  CFU/ml (see 5, p. 110) by seeding medium 523 (20) with cells taken from a fresh culture. Because all agrobacteria do not grow in medium 523, MXY medium (mannitol, 1%; L-glutamic acid, 0.2%; yeast-extract, 0.1%;  $\text{KH}_2\text{PO}_4$ , 0.05%; sodium chloride, 0.02%; magnesium sulfate, 0.02%; pH 7.2) may be preferred. Alternatively, cells can be removed from a colony of the bacterium grown on slants of YDC (see 2), p. 3) or FDA.

Liquid medium 523

Sucrose

Casein (acid hydrolysate; Calbiochem)

Yeast extract

$\text{K}_2\text{HPO}_4$

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

g/L
10.0 g
8.0 g
4.0 g
2.0 g
0.3 g*

\*Dissolve separately in 50 ml distilled water and add prior to autoclaving.

b. Inoculation

Although the economics of time and space must be considered when testing the pathogenicity of an unknown strain, the following is recommended:

- (1) Inoculate two or more plants of each host species per strain.
- (2) Tomato, datura, sunflower and bryophyllum are infected by a large number of Agrobacterium strains. Using combinations of these species, pathogenicity of a strain was identified with 78-94% certainty (2,3). [Note: Conversations with a variety of scientists indicate that tobacco plants, Nicotiana glauca, may be susceptible to a broader range of A. tumefaciens than tomato or sunflower plants. A. tumefaciens AB2173 was originally described as host-specific but later shown to infect N. glauca and members of the cucurbit family (54).]
- (3) Some strains infect only the host species from which they are isolated and may have to be tested on that host.
- (4) Always include controls which consist of: i) plants that are wounded but uninoculated, ii) plants inoculated with a known pathogenic strain of Agrobacterium and iii) plants inoculated with a known nonpathogenic Agrobacterium strain.

A recommended method to test for pathogenicity is to grow three tomato seedlings in each sand-filled cubicle of plastic ice-cube trays and water the plants with any commercial nutrient solution. This allows growth of many plants in a small area. Inoculations can be made on two-week-old seedlings. Water the seedlings carefully to prevent cross contamination. The plants should be kept in a greenhouse at 20-27 C and at a rather high relative humidity.

Wounds which are required for infection can be made in several ways. Make a slit 1-2 mm deep in the stem, crown or root with a scalpel or razor blade or make multiple punctures with a thin, sharp needle. Young and actively growing plants are recommended, or choose the younger parts of older plants. Depending on the type of test, inoculum can be applied (before or after wounding) by a spray, with a transfer loop, hypodermic syringe, pipet, sterile wooden toothpick, or cotton swab.

Allow tumors to develop for at least 3 weeks on herbaceous plants (Plate 2, Fig. 3A) and for at least five weeks on woody plants before assessing for tumor presence. Incubation periods of 4-6 months were required for inoculated incense cedar to develop galls (Moore, L. W., unpublished), and roses may require 18 months (40). If tissue proliferation at the wound of datura plants is still relatively small after 4 weeks, they are recorded as negative because datura plants often form callus in response to wounding (17). Callus on datura usually cease enlargement after 2-3 weeks of growth, whereas bacteria-induced tumors are not self-limiting.

In the absence of greenhouse facilities, disks of carrot roots can be used to test for pathogenicity of *A. tumefaciens* and *A. rhizogenes*. We prefer the carrot root assay (32) (Plate 2, Fig. 3B) to test for pathogenicity of rhizogenic *Agrobacterium* strains. For best results, avoid roots held in prolonged storage. After peeling the root and washing with 95% ethanol, rinse the tissue with 70% ethanol and surface sterilized in a freshly prepared 1:10 dilution of household bleach (containing 5.25% w/v sodium hypochlorite) for 15 minutes. Rinse the root in three changes of sterile water and cut 5 mm-thick slices perpendicular to the axis of the root. Place the slices on moistened sterile filter-paper in petri-dishes and inoculate the tissue with the bacterial suspension. Observe tumor formation after 3 weeks. Sometimes low populations of endogenous bacteria, including *Agrobacterium*, escape the surface sterilant and interfere with the assay. If the pathogenic strains are host-specific to some other host, carrot disks will probably not be infected.

#### 6. OPINE CATABOLISM

Ti and Ri plasmids not only code for pathogenicity but they also code for the catabolism of opines. Opines are unique compounds produced by plant cells which have been transformed by the insertion of T-DNA sequences from Ti or Ri plasmids. There are four opine families: octopine, nopaline, agropine and agrocinopine (51). Octopine and nopaline are the most common opines and are available through Sigma Chemical Co. Opines can serve as a sole carbon and nitrogen source for agrobacteria harboring the appropriate plasmid; different *Agrobacterium* strains and their corresponding Ti or Ri plasmid catabolize different opines. Some strains cannot use the opine as the sole source of both carbon and nitrogen; but they may utilize the opine as a nitrogen or carbon source. To screen for putative opine utilization, a suspension of the strain to be tested is spotted on a basal medium (containing per liter:

$K_2HPO_4$ , 7.2 g;  $KH_2PO_4$ , 2.8 g;  $MgSO_4 \cdot 7H_2O$ , 0.2 g;  $CaCl_2 \cdot 2H_2O$ , 11 mg;  $FeSO_4 \cdot 7H_2O$ , 5 mg;  $MnCl_2 \cdot 4H_2O$ , 2 mg; pH 7.2), gelified with 0.4% gelrite and containing 5 mM of the filter-sterilized opine tested. Controls consist of checking the growth of the strain on the basal medium supplemented with 5 mM glucose and 5 mM ammonium sulfate. Inoculate each medium with a strain known to catabolize the particular opine and with another strain which does not. Because some background growth often occurs on solid media, confirmation should be made in a liquid medium (same as above but without gelrite).

#### 7. TEST FOR AGROCIN SENSITIVITY

The sensitivity of certain strains of *Agrobacterium* to agrocin 84 (28) is another character associated usually with pathogenic strains (14) that are susceptible to the biological control agent *A. radiobacter* K84. However, some agrocin-sensitive strains are not pathogenic on tomato and sunflower (Moore,

L. W., and H. Bouzar, unpublished). To test a strain for sensitivity to agrocin 84 (35), inoculate a spot about 0.5 cm in diameter with K84 on a plate of MG medium (22) and incubate for 48-72 h at 27 C.

<u>Mg medium</u>	<u>per L</u>
mannitol	10.0 g
L-glutamic acid	2.0 g
$\text{KH}_2\text{PO}_4$	0.5 g
NaCl	0.2 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
Biotin (2 µg/l)	1.0 ml*
Agar	15.0 g

\* Prepare stock solutions as follows:

- 1) add 20 mg biotin/100 ml water
  - 2) add 1 ml of above solution to 99 ml of water and filter sterilize
- Adjust pH to 7.0

Prepare a cell suspension of the unknown strain of Agrobacterium (about  $10^8$  CFU/ml) and atomize lightly over the surface of the medium. Incubate 24-48 h until growth of the unknown strain is plainly visible. If the unknown is agrocin 84 sensitive, growth of the unknown strain will be inhibited around the original colony of K84 (Plate 2, Fig. 4). Mutants of agrocin-sensitive strains typically develop in the zone of inhibition (9). For comparison, always include a known agrocin-sensitive strain.

#### 8. PLASMID MINI-SCREEN

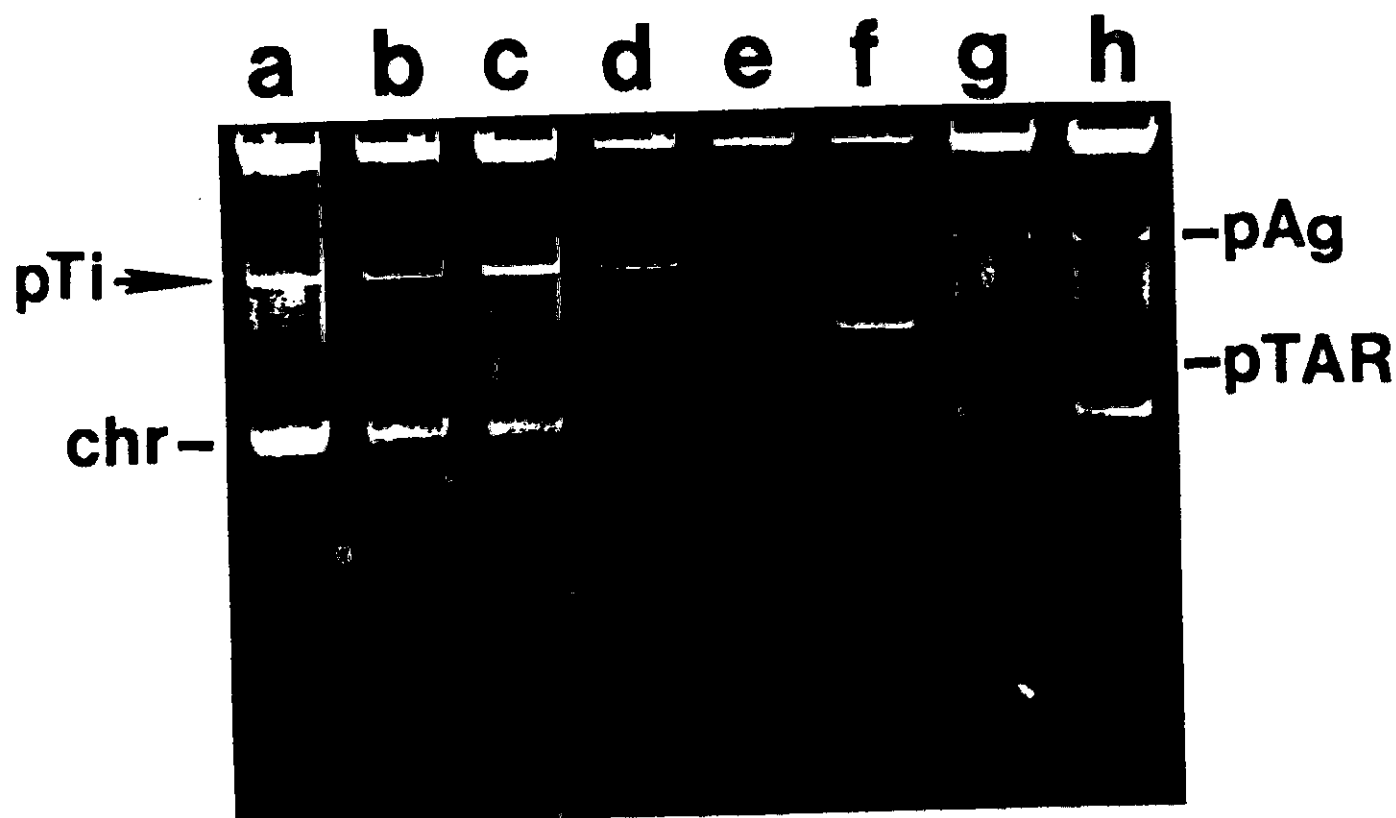
Plasmids confer a number of traits important in virulence maintenance of agrobacteria. Kado and Liu's (21) profile analysis of plasmids provides a means of quickly detecting the presence of large plasmids typically associated with pathogenic strains of Agrobacterium (Fig. 1).

This rapid mini-screen procedure has also been employed for the analysis of small and large plasmids in other Gram-negative and Gram-positive bacteria.

##### Materials:

- a. 2 ml of medium 523 or MGY in screw cap tubes (No. 9826, Corning).
- b. E Buffer: 40 mM Tris-acetate, 2 mM NaEDTA, pH 8.0. This buffer is prepared by titrating 40 mM Tris (Trizma base), in 2 mM NaEDTA with glacial acetic acid to pH 8.0.
- c. Alkaline lysis solution is composed of 3 g Na lauryl sulfate, 5 ml of 3 M NaOH in 100 ml of 0.05 M Tris, pH 12.6. It is important that the pH be accurately measured with a high pH glass electrode.
- d. Phenol-chloroform mixture: Redistilled phenol should be used. Immediately after distillation the phenol can be mixed with an equal volume of reagent grade chloroform. This mixture can be stored at room temperature. Surplus distilled phenol can be stored frozen in polyethylene bottles (do not use nylon bottles since they will be dissolved by phenol). The phenol can be melted in a hot water bath when taken from storage.

- e. For Gram-positive bacteria, a solution of Pronase (2 mg/ml in 2 M Tris-HCl, pH 7.0) is used. Pronase is not necessary for Gram-negative bacteria.



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Fig. 1. Large *Agrobacterium* plasmids isolated by rapid mini-screen procedure (21), and resolved by agarose gel electrophoresis in E buffer. The 200 kb pTi plasmid (arrow) is harbored by tumorigenic strains of *Agrobacterium*. Except for strain 1D1 (=ATCC 15955), a large 300 kb cryptic plasmid (pAg) is observed above the pTi plasmid. The pTi plasmid and other native plasmids are indicated in the following strains: 1D1 (lane a); B6 (lane b); 1D135 (lane d); non-pathogenic grapevine strain 1D1422 containing 44 kb pTAR (lane e); attenuated strain IIBNV6 harboring a 89 kb cryptic plasmid (lane f); nonpathogenic strain R1005 (lane g); nonpathogenic strain from grapevine (lane h).

### Procedure:

- a. Cells are grown to rapid log phase in 2 ml of medium 523 in a screw cap tube (no. 9826, Corning) and harvested by centrifugation (5000g, 5 min). In the same tube, the cell pellet is resuspended thoroughly in 1 ml E buffer. When first attempting to isolate plasmid DNA use this larger volume to obtain greater reproducibility. The cell volume can be reduced by a factor of 10 and all procedures performed in 1.5 ml microfuge tubes once the operator is proficient with the methodology.
- b. To the cell suspension is added 2 ml of lysis solution, and the tube is capped and inverted 20 times to obtain a uniform mixture. Incubate the tube in a water bath at 60 C for 30 minutes.
- c. After incubation, add 6 ml of phenol-chloroform, cap tightly, and shake very briefly (3 sec) to achieve complete emulsification. Centrifuge the mixture at 5000g for 12 min at 4 C. The clear upper aqueous phase contains plasmid DNA. If the upper aqueous phase remains turbid, transfer the contents to a Corex tube (no. 8441, Corning) and centrifuge at 10,000g for 10 min. If the aqueous phase does not clear, too many cells were probably used at the beginning of the procedure.
- d. Transfer the aqueous phase with a plastic Pasteur pipet (DNA sticks to glass pipets) to a tube containing 1 volume of chloroform. Mix briefly and centrifuge to separate the two phases. Transfer the aqueous phase to a clean tube. The preparation can be stored at 4 C for several months.
- e. The plasmid DNA is resolved electrophoretically in a gel of 0.7% agarose prepared in E buffer. Generally, a horizontal electrophoresis apparatus available commercially is used. Mix 40  $\mu$ l of the plasmid preparation with 10  $\mu$ l of tracking dye (0.25% bromocresol purple in 50% glycerol-E buffer) on a sheet of Parafilm (American Can Co., Greenwich, Connecticut). Between 25-50  $\mu$ l of sample is loaded per slot in the gel. Generally a large number of samples can be analyzed simultaneously. Electrophoresis is carried out at 6 volts/cm until the tracking dye reaches the end of the agarose gel.
- f. After electrophoresis the gel is soaked (for 20-30 min.) in 300 ml of ethidium bromide (0.5  $\mu$ g/ml) (wear surgical gloves when handling ethidium bromide because it is a powerful mutagen). The gel is then rinsed with tap water and the plasmid DNA band is visualized by placing the gel over a shortwave ultraviolet light source. Photographs of the gel may be taken with a Polaroid type 55 film.... exposed through a Tiffen 15 orange filter.

### Fine points:

Plasmid DNAs are easily recognized by their sharp bands in the gel (Fig. 1). Linear DNA mainly composed of degraded chromosomal DNA is usually diffuse and migrates to a position in the gel representing a 18 Mdal molecule. If the chromosomal band interferes with a putative plasmid band, the initially lysed sample should be given a longer heat treatment to totally eliminate the band of linear DNA.

Plasmid DNA can be sized best by running known standards in an adjacent well of the same gel simultaneously with the sample. Erwinia stewartii SW2 harbors a set of sized plasmids (10) that have proven useful as standards.

Relatively precise estimates of molecular weights can be obtained by plotting the distance of migration versus the log of the molecular mass of each standard.

Plasmid DNA in mini-screen gels can be blotted onto nitrocellulose membranes (56) and analyzed for sequence homologies by hybridization with a radiolabelled probe made of purified plasmid DNA from the type strain. This analysis yields information on the inter-relationship of a large number of genes.

#### 9. CULTURE PRESERVATION

Agrobacterium strains can be maintained on PDA (supplemented with 0.5%  $\text{CaCO}_3$ ) or MGY for about 6 months at 4 C. Long-term preservation can be achieved when cultures are lyophilized or stored at or below -70 C. For storage at -70 C, grow the cells on slants of MGA for 48 h. Wash the growth from the agar surface with a sterile 30% (v/v) glycerol solution (cryoprotectant). Dispense 1.0 ml of the cell suspension containing at least  $10^8$  CFU/ml into sterile 1.8 ml-cryotubes (A/S Munc, Kamstrup, DK-4000 Roskilde, Denmark) and store the cryotubes in a freezer at -70 C. Precautions must be taken for electrical shutdown or compressor malfunction by having adequate back-up systems. Long-term preservation can also be achieved by freezing Agrobacterium cultures at -179 to -196 C in liquid nitrogen (37). An economical and simple alternative for long-term storage (agar medium) are sterile-water blanks. Two loopful of fresh bacterial growth (agar medium) are carefully placed at the bottom of test-tubes containing sterile distilled water. To minimize evaporation, use screw caps with Teflon liners, wrap the top of tubes in Parafilm (American Can Co., Greenwich, Connecticut) or place the tubes in a plastic bag. These cultures should be kept at 4 C.

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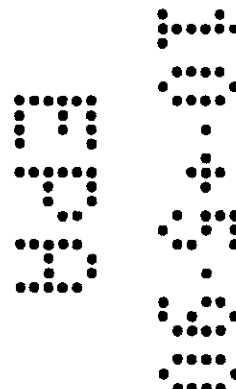
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# 11. Chemicals

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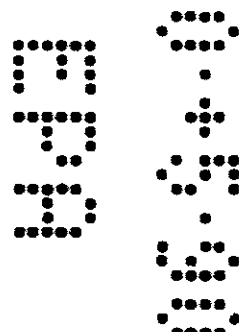
Unless stated otherwise, all the chemicals in this list were obtained from Sigma Chemical Co., St. Louis, MO.

Acetic acid, glacial  
 Adonitol Agar  
 Ammonium nitrate  
 Ammonium phosphate  
 Ammonium sulfate  
 L(-) Arabitol  
 d-Biotin  
 Boric acid  
 Bromocresol purple  
 Bromthymol blue  
 Calcium carbonate  
 Calcium chloride



ChemicalsSource

Caseine, acid hydrolysate	
Chloroform	
Chlorothalonil (Bravo 500)	SDS Biotech Co., Painesville, OH
Crystal violet	
Cupric sulfate	
Cycloheximide	
D-Cycloserine	
EDTA (Ethylenediamine tetraacetic acid), disodium salt	
Erythritol	
Ethanol (ethyl alcohol)	
Ethidium bromide	
Ferric ammonium citrate	
Ferrous ammonium sulfate	
Ferrous sulfate	
Gelrite	Kelco, a division of Merck & Co., Inc., San Diego, CA
D-Glucose	
L(-) Glutamic acid	
Glycerol	
Hydrochloric acid	
$\alpha$ -Lactose	
Lauryl sulfate, sodium salt	Biorad, Richmond, CA
Litmus milk	Difco Laboratories, Detroit, MI
Magnesium sulfate	
Malachite green	
Malonic acid, sodium salt	
Manganese chloride	
Manganese sulfate	
D-Mannitol	
D(+) Maltotriose	
Mucic acid	
Nopaline	
Nutrient agar	Difco
Octopine	
Potassium chloride	
Potassium phosphate dibasic	
Potassium phosphate monobasic	
PDA (Potato dextrose agar)	Difco
Peptone, type III (from soybean)	
Pronase	Calbiochem-Behring, LaJolla, CA
Propionic acid, sodium salt	
Sodium carbonate	
Sodium chloride	
Sodium citrate	
Sodium hydroxide	
Sodium phosphate dibasic	
Sodium phosphate monobasic	
Sodium selenite	
Sodium taurocholate	
Sucrose	



Chemicals	Source
L-Tartaric acid	
Tetramethyl-p-phenylenediamine dihydrochloride	
Trimethoprim	
Triphenyltetrazolium chloride	
Tris (hydroxymethyl)-aminomethane (Trizma base)	
Tris (hydroxymethyl)-aminomethane hydrochloride (Trizma hydrochloride)	
L-Tyrosine	
Yeast extract	Difco
Zinc sulfate	

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Research and Development

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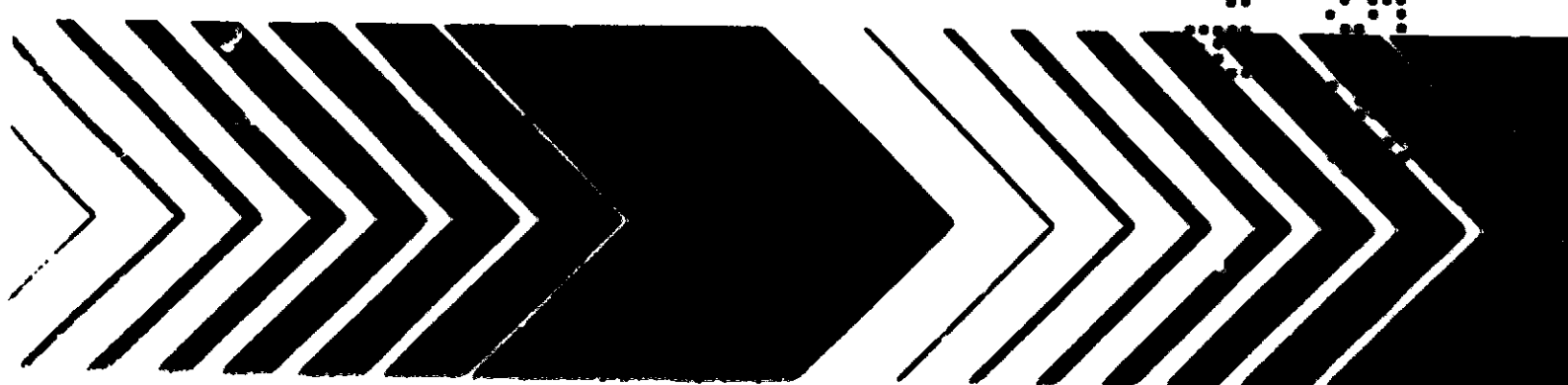


# Review of Progress in the Biotechnology- Microbial Pest Control Agent Risk Assessment Program

*L.W. Moore*

*Study Doc. E-6*

9564



ENVIRONMENTAL FATE OF Agrobacterium radiobacter K84  
RELEASED IN AGRICULTURAL FIELDS

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INTRODUCTION

Agrobacterium radiobacter K84 is an EPA-registered microbial pesticide used successfully in numerous parts of the world for biological control of crown gall disease of plants. The success of this biological control is correlated with the production of agrocin 84, a specific antibiotic against the crown gall pathogen, A. tumefaciens. Genes coding for synthesis of agrocin 84 are located on a conjugative plasmid present in the wild-type strain and can be transferred under laboratory conditions to A. tumefaciens. There is one report of this genetic transfer occurring in a field habitat, resulting in a breakdown of the biological control.

In addition to investigating the possibility of horizontal gene transfer from K84 to a recipient, more information was needed about the persistence and dissemination of K84, or a genetically modified K84, in soil, on target and nontarget plants, and in surface and ground water associated with the agroecosystem. Ecological information about survival and dissemination of K84 has been difficult to obtain because of the lack of reliable methods for detection and monitoring.

Objectives of this research included development of new or improved diagnostic methods to study wild type K84 in the environment. The focus was on more specific, sensitive techniques for detection, isolation, and identification of the target organism among the myriad of other soil microbes, namely the use of a) a strain-specific antiserum to K84 and b) DNA probes of two fragments from the agrocin biosynthetic region. The efficacy and limitations of these methods for ecological studies were compared to traditional methods of selective media, antibiotic-resistant ("marked") strains, and serology while investigating the environmental fate of K84 in agricultural fields.

METHODS

Serology

Strain-specific polyclonal antibodies to A. radiobacter K84 for use in immunoisolation and identification experiments were tested for titer by microprecipitation and specificity by double diffusion and immunofluorescence microscopy.

### Immunoisolation

Preliminary experiments were conducted to determine if K84 could be isolated selectively from aqueous bacterial suspensions with antibodies. Sterile wooden tongue depressors were coated with polyvinyl buterol, and polyclonal antibodies to K84 (1:250 dilution) were adsorbed onto the plastic-coated sticks. The immunoisolation sticks were submerged into a suspension of K84 of known concentration for 1 hr and removed. Then, the bacterial suspension was diluted to determine the number of bacterial cells remaining in the suspension. The sticks were rubbed onto the agar surface of MGY plates to confirm adherence of the bacteria to the antibody-coated sticks.

### Immunoblotting for Identification of K84 Colonies

Immunoblotting is a technique where bacterial colonies are attached to a solid support, reacted with antibodies made to the target strain, and then reacted with a secondary antibody marked with an enzyme. After staining for the marker enzymes, the target organism is cultured on isolation plates and can be identified, enumerated, and reisolated.

### Field Experiments (1988)

Field experiments were planted in May 1988 to compare the survival and biological activity of wild-type strains of Agrobacterium tumefaciens and A. radiobacter, K84 with antibiotic-resistance marked mutants of the same strains on Mazzard cherry seedlings. Plots were located at Ephrata, WA, Moses Lake, WA, Aurora, OR, and Corvallis, OR, and harvested during October.

The number of infected and non-infected cherry seedlings in each plot was recorded. Roots were examined for the presence of galls, and the relative size and number of galls on each root system was recorded. As the seedlings were removed from the soil, root samples were collected from each block at each site, except for the plot at Moses Lake, WA, to determine the rhizosphere populations of the introduced bacteria. Root samples were packed on ice in a cooler and transported back to Corvallis for processing. The tap root was separated from the fibrous roots, rinsed briefly, weighed, and agitated for 30 minutes in a known volume of sterile peptone-phosphate buffer. The suspension was dilution-plated onto semi-selective medium (Kerr 1A for Biovar I or Kerr 2E for Biovar II strains) with some of the medium amended with antibiotics (100 ug/ml rifampicin, 300 ug/ml naladixic acid, or 500 ug/ml streptomycin) for specific recovery of antibiotic-resistant marked strains. Populations were determined after incubation of plates for 7 to 10 days at 25C.

### Field Experiments (1989)

Field experiments were initiated at three locations to examine the survival and spread of the target bacterium, A. radiobacter K84, in the environment.

### Survival of Strain K84 on Cherry Roots

Roots of mazzard cherry seedlings were inoculated with  $10^8$  cfu/ml of strain K84 resistant to 500 ug/ml streptomycin sulfate. Seedlings were planted at the Botany and Plant Pathology Farm near Corvallis, OR, in 5/89. Destructive samples were taken over time to monitor rhizosphere population size of K84 on cherry seedlings.

### Survival of Strain K84 in Fallow Soil

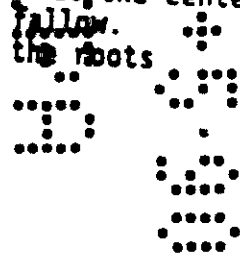
Fallow soil plots at the Botany and Plant Pathology Farm were inoculated in 5/89 with strain K84 resistant to streptomycin or K84 resistant to 100 ug/ml rifampicin, so that the final concentration of bacteria in the soil plot was  $1 \times 10^5$  cfu/g dry weight soil. Soil samples were extracted with phosphate-buffered peptone and dilution plated on Kerr 2E medium amended with the appropriate antibiotics. Near the end of the summer when populations decreased below detectable levels by standard dilution plating techniques, aliquots were either enriched for 24 hours in MGY broth then dilution plated or extracted with wooden tongue depressors coated with strain-specific polyclonal antibodies against strain K84 to capture cells of K84 from the samples.

### Survival of Strain K84 on Annual Ryegrass Roots

Plots seeded with annual ryegrass were inoculated in 5/89 with either strain K84  $sm^r$  or K84  $rf^r$ , so that the final concentration of bacteria added was  $1 \times 10^5$  cfu/g dry weight soil. Root samples were extracted with phosphate-buffered peptone and dilution plated on Kerr 2E medium amended with the appropriate antibiotics.

### Movement of K84 Through the Soil

In the 1988 field tests, K84 could not be detected in fallow soil any further than 5 cm from a cherry seedling to which it had been inoculated at planting time. This may have been due to the inability of the strain to survive in fallow soil or the lack of sensitivity of our detection methods. To determine if K84 could be detected in soil at greater than 5 cm from an inoculated cherry seedling, this year's experiment was modified to include a trap plant. Since our earlier work demonstrated good survival of K84 on grass roots, annual ryegrass was planted in concentric rings at increasing distances (5, 10, 20, 40, 80, and 160 cm) around an inoculated cherry seedling to act as a biological trap for the bacterium as it moves through the soil. Mazzard cherry seedlings inoculated with  $10^8$  cfu/ml K84  $sm^r$  were planted at the center of the rings in 5/89, and the soil between each ring was kept fallow. Ryegrass samples were removed periodically from each ring and the roots assayed for the presence of strain K84.



## RESULTS AND DISCUSSION

### Immunoisolation

When immunoisolation sticks were incubated for one hour in aqueous suspensions of K84 ( $10^2$ ,  $10^3$ , and  $10^4$  CFU/ml), there was a 10 to 100 fold decrease in the number of cells remaining in the suspension. Mixed bacterial populations in soil suspensions posed a problem, because nontarget bacterial cells were physically adsorbed to the sticks, and the sticks had to be streaked to antibiotic-amended media to reisolate the target strain. Methods to improve the procedure are being examined.

### Immunoblotting for Identification of K84 Colonies

Based on fluorescence microscopy, whole bacterial cells from agar colonies adhered best when blotted to loosely woven Whatman #1 filter paper and Whatman 3MM chromatography paper. Whole cells and colonies washed off the other papers and nitrocellulose sheets. The type of medium that the bacteria were grown on affected the adherence of the cells to the papers.

The study of marker enzymes and stains to determine their suitability for immunoblotting whole K84 cells showed that cells immunolabeled with alkaline phosphatase reacted quickly (within 20 min) with the reagents BCIP/NBT--colonies were easily seen as dark blue-black spots. However, non-labeled colonies also reacted slowly with these reagents, and after 6 hours they were indistinguishable from the labeled colonies. Alkaline phosphatase is poorly suited for this technique because of the high background activity from endogenous alkaline phosphatase present in these bacteria.

The use of peroxidase as a marker enzyme avoids some of the non-specific background-staining problems. However, the CNT stain frequently cited in the literature for use with peroxidase reacted poorly with the immunoblots. After 1 hour incubation, the blots were only faintly visible as gray-blue spots, and small colonies (less than 2 mm dia.) were difficult to see. In contrast, the stain AEC reacted well with the immunoblots in the presence of the marker enzyme, peroxidase. Background staining was minimal with the red-colored compound, and small colonies (1 mm dia) were seen easily on the blots. Although very good progress has been made towards developing a more accurate staining procedure, tests are still needed on the specificity of the method and optimization of antibody concentrations and incubation times.

### Harvest of Field Plots (1988)

The background incidence (water-treated controls) of crown gall at all locations was decreased by the wild-type biological control agent radiobacter K84. Average gall size was also reduced by K84 at all locations, with the exception of Moses Lake, WA. Background incidence of crown gall and average tumor size was effectively decreased also at 3 locations by the streptomycin-resistant mutant of strain K84; however, gall incidence at Ephrata, WA, was higher on trees treated with K84<sup>sm</sup> compared to the application of wild type K84. At the Oregon sites approximately  $10^4$  cfu/g root tissue of K84<sup>sm</sup> were recovered from the rhizosphere, whereas at Ephrata, WA,

only 200 cfu/g root tissue were recovered. The decreased ability of K84<sup>sm</sup> to control crown gall at the Ephrata, WA, site may be related to the observation that high populations of the strain were not sustained on root surfaces at that site, or the presence of naturally occurring agrocin-resistant pathogens in the soil.

In general, differences in survival and pathogenicity of *A. tumefaciens* strains seemed to be related more to location and biovar grouping rather than an effect of antibiotic resistance markers. Biovar I strains performed poorly at the sites located in the Willamette Valley in Oregon, i.e., disease incidence and average size of tumors from strains C58, B6 and their antibiotic-resistant derivatives were lower compared to that found in Washington. The two biovar II agrobacteria strains used in the field studies, B49C (*A. tumefaciens*) and K84 (*A. radiobacter*) and their antibiotic-resistant derivatives performed well at all sites. The rifampicin-resistant strain of B49C performed nearly as well as the wild-type strain of the pathogen. The streptomycin-resistant strain of K84 performed comparably to the wild-type strain of the biological control agent. Rifampicin (100 ug/ml) and streptomycin (100 ug/ml) were the most effective of the antibiotic-resistance markers. A few strains of agrobacteria naturally-resistant to these chemicals were recovered from water-treated control seedlings. In contrast, naladixic acid (300 ug/ml) is not recommended for future field recovery studies as numerous strains of ag. bacteria resistant to this antibiotic were recovered from water-treated cherry seedlings.

#### Field Experiments (1989)

##### Survival of Strain K84 on Cherry Roots

During the first week of growth in the field, the population size of the target bacterium, K84, decreased on roots of cherry seedlings by one log unit, but stabilized during the next two weeks at approximately  $1 \times 10^6$  cfu/g root. The population size of K84 on roots remained stable the following four months at approximately  $10^5$  cfu/g root. The target bacterium appears to colonize a niche in the rhizosphere which provides for fairly long-term survival.

##### Survival of Strain K84 in Fallow Soil Plots

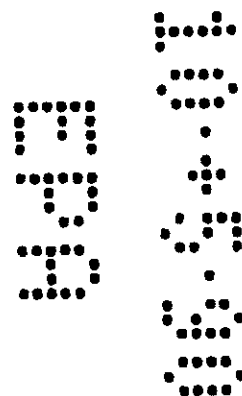
The population sizes of antibiotic-resistant strains of *A. radiobacter* K84 (marked with spontaneous resistance to streptomycin and rifampicin) were similar over the growing season in fallow soil. In contrast to roots, the strains maintained high populations in fallow soil for only 1 week after application and then decreased about 5 log units over the following 15 weeks. By 16 weeks after inoculation of soil plots, K84 <sup>sm</sup> was detected in only 2 of 9 samples by dilution plating. However, immunoisolation and enrichment plating of samples increased detection to 7 out of 9 samples. K84 <sup>rf</sup> was also found sporadically (3 of 9 samples) in dilution-plated soil samples sixteen weeks after inoculation of plots. Treatment of samples by enrichment culture increased detection of these strains slightly to 4 of 9 samples. Treatment of samples with immunoisolation sticks increased detection to 6 of 9 samples. Thus, the immunoisolation procedure increased the sensitivity of detection of the target bacterium in soil over traditional methods.

### Survival of Strain K84 on Annual Ryegrass Roots

Strains K84  $sm^R$  and K84  $rf^R$  maintained a steady population size between  $10^4$  to  $10^5$  cfu/g on roots of annual ryegrass for four months after inoculation. The rhizosphere population sizes on annual ryegrass in the field plots were similar to that observed in studies conducted in the greenhouse. The population size of the rifampicin- and streptomycin-marked strains on annual ryegrass roots were similar also, which indicates that either antibiotic-resistance marker can be used as a suitable standard of comparison to newer detection methods for ecological studies of strain K84 under field conditions.

### Movement of K84 Through the Soil

K84 was detected on grass seedlings 5 cm away from the point source of inoculum one and two weeks after planting K84  $sm^R$  inoculated cherry seedlings at the center of rings of grass. Five weeks after planting, strain K84 was found 10 cm away from the seedling and in one case, at a distance of 20 cm from the cherry plant. Eight and twelve weeks after planting, strain K84 was detected in ryegrass rings 40 cm distal to the inoculated seedlings. In contrast, last field season, five centimeters was the maximum distance we detected K84 in soil around inoculated seedlings. Importantly, the use of annual ryegrass as a biological trap plant increased our ability to trace this bacterium through the environment.



Study Doc. E-7

**Saprosaic Nematodes as Carriers and Disseminators of  
Plant Pathogenic Bacteria**

**BY AROON CHANTANAO AND HAROLD J. JENSEN**

933371

## Sapropoic Nematodes as Carriers and Disseminators of Plant Pathogenic Bacteria<sup>1</sup>

AROON CHANTANAO<sup>2</sup> AND HAROLD J. JENSEN

**Abstract:** The plant pathogenic bacteria *Agrobacterium tumefaciens* (Smith and Townsend) Conn. (strain 5-14 Deep), *Erwinia amylovora* (Burill) Winslow et al., *E. carotovora* (Jones) Holland and *Pseudomonas phaseolicola* (Burk.) Dows. (ICPB-PM3) and the red-pigmented non-pathogen *Serratia marcescens* Bizio were hosts for the sapropoic nematode *Pristionchus lheritieri* (Maupas, 1919) Paramonov. Viable bacteria survived passage through the nematode and produced typical colonies on nutrient agar plates. Female nematodes ingested more bacterial cells and retained them longer than did males. It was hypothesized sapropoic nematodes may disseminate pathogenic bacteria to new infection courts.

Most investigators conclude that sapropoic nematodes feed on bacteria or by-products of bacterial metabolism. Occurrence of this group of nematodes in association with decomposing plant tissue or in soil samples is commonly recognized. Interrelationships of sapropoic nematodes and plant pathogenic bacteria occasionally have been reported. For example, Steiner (3) suggested in 1933 that a sapropoic nematode, *Pelodera* (*Rhabditis*) *lambdiensis* (Maupas) Dougherty may carry a mushroom pathogen, *Pseudomonas tolaasii* Paine, in its pharynx or intestine. Jensen (2) recently reported *Pristionchus* (*Diplogaster*) *lheritieri* (Maupas) Paramonov, *Panagrellus redivivus* (Linn.) Goodey, *Panagrolaimus subelongatus* (Cobb) Thorne, and *Rhabditis* spp. ingested and voided viable spores of various plant pathogenic fungi and a plant pathogenic bacterium, *Pseudomonas syringae* Van Hall.

Ingestion and voiding (defecation) of viable phyto-bacteria by sapropoic nematodes may be of some significance in dissemination and survival of bacterial pathogens. This investigation is concerned with associations

of a sapropoic nematode and 4 species of plant pathogenic bacteria.

### MATERIALS AND METHODS

*Pristionchus lheritieri*, originally isolated from a decaying carrot approximately three years ago and maintained in the laboratory on cultures of *Pseudomonas* sp., was selected as the test vector. Pure cultures of *Agrobacterium tumefaciens* (Smith and Townsend) Conn Strain 5-14 Deep (obtained from I. W. Deep, Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon), *Erwinia amylovora* (Burill) Winslow et al. (obtained from Carolina Biological Supply Company, Powell Laboratories Division, Gladstone, Oregon), *Erwinia carotovora* (Jones) Holland (obtained from Carolina Biological Supply Company, Powell Laboratories Division, Gladstone, Oregon), *Pseudomonas phaseolicola* (Burk.) Dows. ICPB-PM3 (obtained from M. P. Starr, Curator, International Collection of Phytopathogenic Bacteria, University of California, Davis, California) were used as test organisms to demonstrate ingestion and survival.

The following is a brief account of a method developed to exchange the natural bacterial flora of the nematode's alimentary tract as suggested by Chantanao (1). Gravid females were surface-sterilized 20 min in 20

TABLE 1. Plate counts of *Pristionchus lheritieri*.

Period of Starvation (hr)	Rep. 1
0	49,000
3	25,375
6	21,000
9	8,712
12	6,812
15	5,350
18	2,550
21	1,437
24	364
27	101
30	44
33	0

\* Average of four nematodes.

ppm chlorine then to nutrient agar (3 g of peptone, 15 g of agar water) containing 10% hydrochloride. Eggs plates were transferred containing the red-*Serratia marcescens* H. Hayes, Department Oregon State University. This bacterium is utilized in contamination when oil occur. Gravid female plates were surface-sterilized to nutrient agar plates containing tetracycline hydrochloride. Eggs from the nutrient agar plates were transferred to a plate containing complete the flora.

A different procedure for determining the number of bacteria ingested and this bacterium in the culture time in A. 11 is described depending upon

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TABLE 1. Plate counts of *Agrobacterium tumefaciens* ingested and defecated during 24 hr by female *Pristionchus lheritieri*, after various periods of starvation.

Period of Starvation (hr)	Number of Bacteria Per Nematode*				Mean	Defecation of Bacteria
	Rep. 1	Rep. 2	Rep. 3	Rep. 4		
0	49,000	43,625	32,250	57,000	46,219	+
3	25,375	34,750	27,875	31,625	30,406	+
6	21,000	24,250	9,375	8,750	15,844	+
9	8,712	6,987	7,262	7,237	7,550	+
12	6,812	6,100	6,700	5,600	6,303	+
15	5,350	4,675	5,225	4,862	5,028	+
18	2,550	3,850	2,975	3,637	3,252	+
21	1,437	1,837	1,987	1,925	1,797	+
24	364	402	291	252	327	+
27	101	107	92	122	106	+
30	44	19	54	36	38	-
33	0	0	0	0	0	-

\* Average of four nematodes each.

nd Townsend) Conn. (Jones) Holland and non-pathogen *Serratia* Maupas, 1919) Paratypical colonies on red them longer than mic bacteria to new

and 4 species of

## METHODS

originally isolated approximately three in the laboratory as sp., was selected cultures of *Agro-Smith* and Town- Deep (obtained ent of Botany and State University, *is amylovora* (Bur- ned from Carolina ry, Powell Labora- , Oregon), *Erwinia* nd (obtained from Company, Powell adstone, Oregon), s (Burk.) Dows. M. P. Starr, Cura- on of Phytopatho- ty of California, used as test orga- tion and survival. ief account of a hange the natural atode's alimentary tanao (1). Gravid ized 20 min in 20

ppm chlorine then transferred to plates of nutrient agar (3 g of beef extract, 5 g of peptone, 15 g of agar in one liter of distilled water) containing 1000 ppm of tetracycline hydrochloride. Eggs deposited on these plates were transferred to nutrient agar plates containing the red-pigmented bacterium, *Serratia marcescens* Bizio (obtained from H. Hayes, Department of Microbiology, Oregon State University, Corvallis, Oregon). This bacterium is utilized to indicate contamination when other than red colonies occur. Gravid females from the *S. marcescens* plates were surface-sterilized and transferred to nutrient agar plates containing tetracycline hydrochloride as previously described. Eggs from these plates were placed upon nutrient agar plates. If no carry-over contamination (indicated by red colonies) was observed, emerging larvae were transferred to a plate containing different bacteria to complete the flora exchange.

A different procedure was used to determine the number of *Agrobacterium tumefaciens* ingested and the survival time of this bacterium in the alimentary tract. Accession time in *A. tumefaciens* cultures varied depending upon the type of experiment.

Nematodes used to determine the survival time of ingested bacteria were limited to a standard feeding period of 24 hr. After feeding, the nematodes were removed, surface-sterilized 20 min in 20 ppm chlorine and transferred to nutrient agar plates containing tetracycline hydrochloride. Many bacteria, including test bacteria, were unable to grow in this medium thus eliminating the source of additional food for the nematodes. At 3-hr intervals several males and females were removed and placed in 20 ppm chlorine for 20 min. Following this treatment, four replicate groups (each containing four nematodes) of males and of females were transferred to nutrient agar plates (one plate for each group). Similar groups were aseptically crushed in 1 ml distilled water, the volume increased to 10 ml and 1 ml carried through a standard dilution series ( $10^{-2}$  to  $10^{-5}$ ) transferred to duplicate nutrient agar plates. Colony counts were made after incubation 48 hr at 25 C.

## RESULTS AND DISCUSSION

*Pristionchus lheritieri* from laboratory cultures upon *Pseudomonas* sp. thrived on the five different bacteria, including the red-

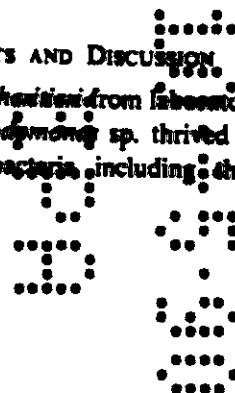


TABLE 2. Plate counts of *Agrobacterium tumefaciens* ingested and defecated during 24 hr by male *Pristionchus lheritieri*, after various periods of starvation.

Period of Starvation (hr)	Number of Bacteria Per Nematode*				Mean	Defecation of Bacteria
	Rep. 1	Rep. 2	Rep. 3	Rep. 4		
0	10,375	7,615	12,375	9,375	9,937	+
3	376	384	522	600	491	+
6	187	171	235	252	211	+
9	79	156	112	141	122	+
12	12	16	32	39	25	-
15	0	0	0	0	0	-

\* Average four nematodes each.

pigmented *S. marcescens* and the plant pathogens *A. tumefaciens*, *E. amylovora*, *E. carotovora* and *P. phaseolicola*. At least some bacteria of all species survived passage through the nematode's digestive tract and produced typical growth upon nutrient agar plates. After feeding 24 hr, female nematodes defecated viable *A. tumefaciens* and *P. phaseolicola* for an additional 27 hr and *E. amylovora* and *E. carotovora* were defecated for 21 hr. Males did not defecate viable bacteria after 9 hr.

Interrelationship studies of *P. lheritieri* and the crown gall bacterium indicated ingestion of an average of about 46,000 bacterial cells in 24 hr by female nematodes (Table 1) and 10,000 bacterial cells by males (Table 2). The smaller number ingested by males probably reflects their smaller size and food requirements as well as less aggressive feeding habits. In either sex, the maximum accumulation of bacterial cells occurred during the initial 24-hr feeding period. In carrier nematodes denied access to food by placing them on antibiotic-supplemented media, the number of recoverable bacterial cells decreased rapidly to zero. Thus survival or persistence in the carrier nematode was limited to 30 hr in the female (Table 1) and 12 hr in the male (Table 2). A prolonged cessation of the food supply interrupted defecation and probably many other body functions

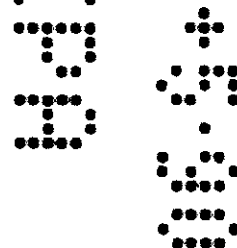
which may affect the persistence of bacteria in the carrier nematode.

Bacteria or by-products of bacterial metabolism support enormous populations of saprozoic nematodes in laboratory cultures. Although numerous typical colonies grew from defecations, we did not determine the fate of all ingested cells including those probably used for sustenance. The superabundance of bacteria available for food in these experiments may have exceeded the digestive capacity of the nematode thus allowing more cells to pass through unharmed than if fewer bacteria were ingested.

Nematodes also can disseminate bacterial pathogens which adhere to their external body surface as well as by ingestion and defecation. Thus the important role of saprozoic nematodes in this interrelationship appears to be one of dissemination.

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